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(54) **Drug delivery system involving interaction between protein or polypeptide and hydrophobic biodegradable polymer.**

(57) A drug delivery system for controlled release of a protein or polypeptide comprising a hydrophobic biodegradable polymer and a protein or polypeptide. A physical interaction is present between the polymer and the protein or polypeptide, thus, allowing protection and controlled release of the protein or polypeptide in-vivo. The drug delivery system may be prepared by a polymer precipitation technique or a microsphere technique.

EP 0 467 389 A2

BACKGROUND OF THE INVENTION

(1) Field of the Invention

The present invention relates generally to the field of biodegradable polymers for the controlled release of biologically active agents therefrom. More particularly, the present invention relates to a process for preparing hydrophobic biodegradable polymers of controlled size in which there is a physical interaction with the protein or polypeptide incorporated therein. Such an interaction promotes incorporation of the protein or polypeptide into the polymer matrix and allows for protection and controlled release of the protein or polypeptide from the polymer.

(2) Background of the Prior Art

A wide variety of microencapsulation drug delivery systems have been developed heretofore for the rate controlled release of therapeutic agents or other agents. For instance, considerable research has been devoted to incorporating therapeutic agents into polyesters such as poly-ϵ-caprolactone), poly(ϵ-caprolacto ne-Co-DL-lactic acid), poly(DL-lactic acid), poly(DL-lactic acid-Co-glycolic acid) and poly(ϵ-caprolactone-Co-glycolic acid) in which release was diffusion controlled. See, for example, Pitt, C.G., Gratzl, M.M., Jeffcoat, A.R., Zweidinger, R., Schindler, A.," Sustained Drug Delivery Systems. II. Factors Affecting Release Rates from Poly(ϵ-caprolactone) and Related Biodegradable Polyesters", J. Pharm. Sci., 68, 1534 (1979). These systems were fabricated as films and capsules and the results suggest that the devices can be prepared to erode after release of the drug is essentially complete. Degradation of the polyesters has been reported to proceed by random hydrolytic cleavage of ester linkages by an autocatalytic process with the rate of chain cleavage being influenced by chemical and morphological factors.

Sustained release systems of antimalarial agents and sulfadiazine in glycolic-lactic acid copolymers have also been reported. Wise, D.L., Gesser, J.D., McCormick, G.J., "Sustained Release of a Dual Anti-malarial System", J. Pharm. Pharmacol., 31, 201 (1979). Wise, D.L., McCormick, G.J., Willett, G.P., Anderson, L.C., Howes, J.F., J. Pharm. Pharmacol., 30, 686 (1978). Methods reported by the foregoing investigators involved dissolving the agents in a suitable solvent and either spray drying or casting films according to usual methods and evaporating the solvent. Various narcotic antagonists and steroids have been incorporated in films and implanted in rats (e.g., see Woodland, J.H.R., Yolles, S., Blake, D.A., Helrich, M., Meyer, F.J., "Long-Acting Delivery Systems for Narcotic Antagonists: I", J. Med. Chem., 16, 897 (1973), Jackanicz, T.M., Nash, H.A., Wise, D.L., Gregory, J.B., "Polylactic Acid as a Biodegradable Carrier for Contraceptive Steroids", Contraception, 8, 227 (1973). Anderson, L.C., Wise, D.L., Howes J.F., "An Injectable Sustained Release Fertility Control System", Contraception, 13, 375 (1976) and incorporated into particles injected subcutaneously [Yolles, S., "Time-Release Depot for Anticancer Drugs: Release of Drugs Covalently Bonded to Polymers", J. Parent. Drug Assoc., 32, 188(1978)]. The release of a number of anti-tumor agents has been evaluated in implantable systems as reported in [Yolles, S., "Time Release Depot for Anticancer Drugs: Release of Drugs Covalently Bonded to Polymers", J. Parent. Drug Assoc., 32, 188 (1978)], and the antibiotic Mitomycin C has been encapsulated in microspherical carriers of gelatin and administered intravenously [Yoshioka, T., Hashida, M., Muranishi, S., and Sezaki, H., "Specific Delivery of Mitomycin C. to Liver, Spleen and Lung: Nano- and Microspherical Carriers of Gelatin", Intern. J. Pharm., 81, 131 (1981)] and the effect of size on in vivo distribution and the potential for antibiotic targeting was discussed. The size distribution of the microspheres (i.e., 5 to 30 μm) reported in the last mentioned publication was very broad, especially for intravenous administration. Recently the in-vitro release of local anesthetics from polylactic acid spheres prepared by a solvent evaporation process has, likewise, been reported [Wakiyama, N., Kaxuhiko, J., Nakano, M., "Influence of Physicochemical Properties of Polylactic Acid on the Characteristics and In Vitro Release Patterns of Polylactic Acid Microspheres Containing Local Anesthetics", Chem. Pharm. Bull., 30, 2621 (1982)]. The patterns of release from these polylactic acid spheres were characterized by the various degrees of degradation of the polymer as well as solubilities of loaded drugs, although no attempt was apparently made to evaluate this parameter. Additionally, it is apparent that the solubility of the drug played an important role in the rate and extent of release. Scanning electron photomicrographs also revealed varying degrees of erosion and deformation of the spheres after release.

It will be seen from the foregoing that while the controlled release delivery of pharmaceuticals or other agents from heretofore described polymeric systems has been principally limited to oral, topical or implantable systems in which the considerations relative to pore size and/or cell size within the carrier matrix as well as the overall dimensions of the microspheres to be administered along with the rate of

release and the relative absorption rate from a bioavailability standpoint are distinctly different from the evaluation parameters involved in the utilization of these microsphere delivery systems for parenteral, i.e., intravenous, intraarterial, intramuscular, subcutaneous, intraocular or inhalation administration routes to which the present invention is particularly applicable.

For instance, U.S. Patent No. 4,818,542 describes a controlled release drug delivery system comprised of a spherical microprocess polymeric network of interconnecting channels.

Further, the use of proteins and peptides as therapeutic agents has been recognized and their position within the pharmaceutical armamentarium is growing due to their increasing availability. This availability is primarily due to recent advances in genetic engineering and biotechnology. Unfortunately, the use of proteinaceous drugs by conventional routes of administration is generally hampered by a variety of delivery problems. Nonparenteral routes of administration, i.e., oral and percutaneous, are inefficient primarily due to poor absorption of proteinaceous drugs into the bloodstream and degradation of such drugs in the gastrointestinal tract. Rapid proteolytic inactivation of the proteinaceous drug also occurs when the drug is administered parenterally thus decreasing its bioavailability. In addition, when administered by the parenteral route, the host's immune system is activated thereby potentially setting off a series of undesirable immune reactions.

In view of the foregoing, considerable effort has been devoted to developing alternative systems for parenteral delivery of peptides and proteins to obviate the problems associated with prior art administration techniques. For instance, implantable devices have been cast or molded from poly-(hydroxyethyl)-methacrylate, polyvinyl alcohol, ethylenevinylacetate copolymer (EVA) and silicone elastomer. Macromolecular drugs have been embedded in those devices. A typical method of preparation involves suspending a powder of a macromolecular drug such as a solid protein or peptide in a solution containing the polymer. The entire composition is then case or molded into the desired size and shape either by evaporating the solvent or by vulcanization. A sustained release of macromolecules from these devices has been demonstrated. The simplicity of the foregoing prior art method is its primary advantage.

To avoid the foregoing difficulties, U.S. Patent No. 4,741,872 discloses a method for preparing biodegradable microspheres having a three-dimensional network in which biologically active macromolecular agents are physically entrapped therein.

A number of other types of protein/polymer systems are known in the art. For instance, U.S. Patent Nos. 3,843,446, 3,977,941 and 4,601,981 discloses the preparation of enzymatically active protein-enzyme complex membrane by treating another protein membrane with an aqueous solution of an enzyme. The membranes are used to effect enzymatic reactions.

U.S. Patent No. 3,972,776 discloses the preparation of enzymatically active protein-whole microbial cell complex membranes suitable for effecting enzymatic reactions by forming a dispersion containing synthetic or natural protein macromolecules and whole microbial cells, casting a membrane from the dispersion and drying the membrane. The membranes may also be formed by electro-codeposition from a dispersion containing the macromolecular and cells.

U.S. Patent No. 4,758,342 relates to a hyperfiltration membrane containing a supporting layer and a separation layer.

U.S. Patent Nos. 4,494,944 and 4,557,855 disclose a surface active agent comprised of lignin sulfonic acids, and optionally, an alkylaryl sulfonic free acid with at least ten carbon atoms and eight polypeptides having a molecular weight of about 2,500 to about 15,000.

U.S. Patent Nos. 4,585,797 and 4,591,501 disclose a flexible continuous film which is comprised of a physical admixture of a polypeptide, a plasticizer and a film-forming flexible polymer, when the film is moistened, the polypeptide exudes therefrom.

U.S. Patent No. 4,873,033 relates to a hyperfiltration membrane containing a supporting layer and a separation layer. The separation layer consists of a crosslinked monomolecular film of molecules, the molecules of the separation layer in the uncrosslinked state being surfactants or surfactant-like lipoids containing at least one hydrophobic chain and at least one hydrophilic group. The surfactant-like lipoid molecules are spread out under a certain spreading pressure or occupy an average space over the surface of an aqueous solution or at the interface between an aqueous solution and a liquid immersible therewith.

U.S. Patent No. 4,897,444 relates to an immobilized fluorogenic substrate. The substrate has the structure

$$R_1 - NH - R_4 - R_2 - R_3$$

wherein R_1 represents an enzyme-specific oligopeptide, R_2 represents a spacer group which is a methylene-carboxyloxy, a methylene carboxamido or a methylenesulfonamido group attached to a poly-

methylene chain which itself has a functional group suitable for coupling with a polymer; R_3 represents a biologically inert polymer; and R_4 represents a fluorogenic moiety.

GB 2 207 050 discloses a composition comprising an aqueous solution of a drug and a glucose polymer mixture which includes at least 50% by weight of glucose polymers of D.P. greater than 12. The composition is introduced into the peritoneal cavity. The glucose polymers act as osmotic agents during peritoneal dialysis.

EP 0 354 714 discloses a pharmaceutical composition for affecting tissue redistribution of bioactive peptides and proteins which are normally bound to glycoaminoglycans, and for mimicking the action of glycoaminoglycans in biological interactions. The composition comprises a pharmaceutically acceptable polymeric compound having monomeric units and a molecular weight between 1,000 and 20,000 Daltons, wherein each monomeric unit contains between three and about 10 aromatic rings.

EP 0 187 547 relates to polymeric drugs comprising an inert synthetic polymeric carrier covalently attached to low molecular weight bioactive molecules. Drug delivery is somewhat targeted because uptake is restricted to cells capable of a substrate selective mechanism known as pinocytosis.

In spite of the numerous teachings of the prior art, the prior art drug delivery systems still have some significant disadvantages and commercialization has been difficult to achieve especially with respect to sufficient drug loading, reproducibility of product specifications and scale-up.

SUMMARY OF THE INVENTION

It is, therefore, the object of this invention to provide one or more processes for the incorporation of polypeptides and proteins into a hydrophobic biodegradable polymer to provide a stable formulation and to achieve protection and controlled release of the polypeptide or protein from the polymer in vivo.

It is another object of the present invention to provide for a drug delivery system itself which allows for controlled release of the polypeptide or protein from the polymer in vivo, wherein said incorporation, protection and controlled release are due to the physical interaction between the polypeptide or protein and the hydrophobic biodegradable polymer.

A still further object of the present invention is to provide a microspherical drug delivery system which allows targeting of drugs or other agents to specific host tissues or cells via injection or inhalation providing high localized concentrations, sustained activity, systemic administration and treatment, thereby minimizing undesirable systemic effects of toxic drugs administered in the native form.

These and similar objects, advantages and features are accomplished according to the methods and compositions of the following description of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a description of the precipitation method of preparing the drug delivery system of the present invention.

Fig. 2 is a description of the microsphere method of preparing the drug delivery system of the present invention.

Fig. 3 is a graph depicting the interaction of salmon calcitonin (sCT) with polyglycolic acid, polylactic acid and copolymers of glycolide and L-lactide at varying molar concentrations of polymer.

Fig. 4 is a graph of the size distribution of salmon calcitonin and polyglycolic acid microspheres.

Fig. 5 is a graph of the release of salmon calcitonin from polyglycolic acid Mw = 100,000 and salmon calcitonin microspheres prepared by freeze drying. The targeted drug load was 10%.

Fig. 6 is a graph of the release of salmon calcitonin from a polyglycolic acid-salmon calcitonin precipitate. The targeted drug load was 10%.

Fig. 7 is a graph of the serum calcium concentration over time of a salmon calcitonin/microsphere drug delivery system.

DESCRIPTION OF THE PREFERRED EMBODIMENTS OF THE INVENTION

A variety of hydrophobic biodegradable polymers are suitable in the drug delivery system of the present invention. Such polymers are well-known to those of ordinary skill in this art. Suitable polymers include polyesters, polyorthoesters and polyanhydrides.

The polymer may comprise copolymeric and homopolymeric polyesters containing hydrolyzable inter linkages which are, therefor, biodegradable. Typically preferred of such polyesters are polyglycolic (PGA) and polylactic (PLA) acids, and copolymers of glycolide and L-lactide (PGL). The aforementioned polyesters

are particularly suited for the methods and compositions of the present invention by reason of their characteristically low human toxicity and virtually complete biodegradability. Of course, it will be understood that the particular polyester or other polymer, oligomer, copolymer, and the like, utilized in the present invention is not critical and a variety of hydrophobic biodegradable polymers may be utilized as a consequence of the novel processing methods of the invention which yield the desired drug delivery system, irrespective of the source of polymer utilized.

Accordingly, other biodegradable or bioerodable polymers or copolymers evidencing the necessary low degree of toxicity suitable for use in the present invention include, for example, gelatin, agar, starch, arabinogalactan, albumin, collagen, natural and synthetic materials or polymers, such as poly(ε-caprolactone), poly(ε-caprolactone-Co-lactic acid), poly(ε-caprolactone-Co-glycolic acid), poly(β-hydroxy butyric acid), polyethylene oxide, polyethylene, poly(alkyl-2-cyanoacrylate), (e.g., methyl, ethyl, butyl, and the like), hydrogels (e.g., poly(hydroxyethyl methacrylate poly-hydroxyethyl methacrylate), polyamides (e.g., polyacrylamide), poly(amino acids)(i.e., L-leucine, L-aspartic acid, β-methyl-L-aspartate, β-benzyl-L-aspartate, glutamic acid and the like), poly(2-hydroxyethyl-DL-aspartamide), poly(ester urea), poly(L-phenylalanine/ethylene glycol/1,6-diisocyanatohexane), poly(methyl methacrylate), 3,9-bis methylene-2,4,8,10-tetraoxaspirol [5,5] undecane, 1,6-hexadiol polyorthoester, poly(bis-p-carboxyphenoxypropane anhydride), ethylene-vinylacetate copolymer (EVA), polyvinyl alcohol (PVA) and silicone elastomer.

The foregoing exemplary natural and synthetic polymers suitable for use in the present invention are, of course, either readily available commercially or are obtainable by condensation polymerization reactions from the suitable monomers or, comonomers or oligomers. For instance, homopolymers and copolymers of glycolic and lactic acids can be prepared by direct polycondensation or by reacting glycolide and lactide monomers as disclosed by Gilding, D.K., Reed, A.M., "Biodegradable Polymers for Use in Surgery - Polyglycolic/Poly(lactic acid) Homo- and Copolymers: 1", Polymer, 20, 1459 (1979).

Any protein or polypeptide is suitable in the practice of the present invention. Biologically active proteins or polypeptides for use in the present invention are proteins or polypeptides of relatively small molecular weights. Exemplary preferred biologically active polypeptides for use herein are calcitonin, insulin, angiotensin, vasopressin, desmopressin, LH-RH (luteinizing hormone-releasing hormone), somatostatin, glucagon, somatomedin, oxytocin, gastrin, secretin, h-ANP (human atrial natriuretic polypeptide), ACTH (adrenocorticotropic hormone), MSH (melanocyte stimulating hormone), beta-endorphin, muramyl dipeptide, enkephalin, neurotensin, bombesin, VIP, CCK-8, PTH (parathyroid hormone), CGRP (calcitonin gene related peptide), endothelin, TRH (thyroid releasing hormone), growth hormones like erythropoietin, lymphokines like macrophage stimulating factor, and the like. The various polypeptides for use herein include not only the naturally occurring polypeptides themselves but also pharmacologically active derivatives and analogs thereof. Thus, for example, calcitonin intended for use in the present invention includes not only naturally occurring products such as salmon calcitonin, human calcitonin, porcine calcitonin, eel calcitonin and chicken calcitonin, but also analogs such as [$Asu^{1,7}$]-eel calcitonin elcatonin, a product of Toyo Jozo Company, Ltd., as well. Similarly, LH-RH for use herein includes not only the naturally occurring product but also the pharmaceutically active derivatives and analogs thereof such as described in various patents and publications referenced hereinabove, e.g., Matsuzawa et al U.S. Patent No. 3,917,825. Especially preferred polypeptides for use in the present invention include calcitonin, insulin, ACTH, LH-RH, PTH, CGRP, somatostatin and somatomedin. Calcitonin is the most preferred.

Biodegradable synthetic polypeptides include poly-(N-hydroxyalkyl)-L-asparagine, poly-(N-hydroxyalkyl)-L-glutamine, copolymers of N-hydroxyalkyl-L-asparagine and N-hydroxyalkyl-L-glutamine with other amino acids.

Definitions or further description of any of the foregoing terms and phrases are well known in the art and may be found by referring to any standard biochemistry reference text such as "Biochemistry" by Albert L. Lehninger, Worth Publishers, Inc. and "Biochemistry" by Lubert Stryer, W.H. Freeman and Company, both of which are hereby incorporated by reference.

The amount of the biologically active peptide in the drug delivery system of the present invention will vary, depending upon the particular polypeptide employed, but will be an amount sufficient to elicit the desired pharmacological effect. Thus, for example, when the selected polypeptide is calcitonin, such will be present in an amount sufficient to treat a condition such as Paget's disease or hypercalcemia or osteoporosis. A typical preparation may contain, for example, from about 0.01 to about 0.04 I.U./mg for porcine calcitonin. In the case of insulin, an amount sufficient to control blood sugar levels and thus to treat diabetes will typically be employed; in the case of LH-RH or analog thereof, an amount sufficient to treat diabetes will typically be employed; in the case of LH-RH or analog thereof, an amount sufficient to treat various disorders of the female reproductive system, an amount sufficient to have a contraceptive effect, or an amount sufficient to elicit any other known biological response to LH-RH will be used, in the case of

PTH, CGRP, somatomedin or analog thereof, an amount sufficient to treat various disorders of bone metabolism will be used; and so on for the other biologically active peptides contemplated by the present invention. Thus, the amount of protein or polypeptide useful in the drug delivery system of the present invention is an amount sufficient to achieve the desired therapeutic effect. For guidance, reference may be made to any standard reference text such as Goodman and Gilman, The Pharmacological Basis of Therapeutics.

In order to improve the properties and appearance of the drug delivery system of the present invention, one or more excipients, coloring agents, isotonic agents, antioxidants, and the like, may be added to the drug delivery system, for example, excipients such as starch, dextrin, mannitol, sorbitol, cyclodextrin and tragacanth, coloring agents such as beta-carotene, red color No. 2 and blue color No. 1, isotonic agents such as sodium chloride and glucose, and antioxidants such as ascorbic acid and erythorbic acid and their salts or esters. Actual methods of preparing such dosage forms are known or will be apparent, to those skilled in the art. For example, see Remington's Pharmaceutical Sciences, 17th edition, 1985, ed. Alfonso R. Gennaro, Mack Publishing Company, Easton, Pennsylvania 18042.

The nature of the excipient(s) will preferably aid in the manufacture of the dosage form selected. Certain dosage forms provide a more extended release of the biologically active protein or polypeptide. These extended release dosage forms are particularly useful and offer increased flexibility in the administration of the protein or polypeptide.

An important feature of the present invention is the fact that there is a physical interaction between the hydrophobic biodegradable polymer and the protein or polypeptide of the present invention. That physical interaction may be characterized as an affinity or as some type of association or interaction between the polymer and the protein/polypeptide.

The physical interaction or adsorption is not clearly understood but it may be characterized somewhat by what it is not. The interaction does not appear to be chemical in nature, i.e., it is not a covalent bond, hydrogen bond or the like. This deduction is on the basis of Differential Scanning Calorimetry, Infrared Spectroscopy, Fourier Transform Infrared Spectroscopy, Raman Spectroscopy and Fourier Transform Raman Spectroscopy. While not wishing to be bound by any theory, the present inventor believes that the interaction is hydrophobic in nature and involves the amino acid chain linkages. Briefly, it may be depicted as an equilibrium mechanism:

$$\text{Calcitonin} + \text{Polymer} \rightleftarrows \text{Calcitonin-Polymer}$$

Such a mechanism would allow for incorporation of the protein or polypeptide into the polymer matrix as well as allowing its release from the matrix when localized in a body compartment in which the released protein or polypeptide diffuses from the site.

The drug delivery system of the present invention may be prepared by any procedure which allows for the formation of a physical interaction between the hydrophobic biodegradable polymer and the protein or polypeptide. Two such processes may be referred to as the polymer precipitation technique or the microsphere technique.

In the polymer precipitation technique, the polypeptide and polymer are mixed together with a suitable solvent to form a homogeneous liquid state as depicted in Fig. 1.

Any organic or inorganic solvent may be used as long as both the polypeptide and polymer are soluble in the solvent and the solvent does not degrade or adversely affect the polymer or the polypeptide.

Suitable solvents include but are not limited to methylene chloride, hexafluoroacetone, hexafluoroisopropanol, acetonitrile, hexane, cyclohexane and the like.

The preferred solvents are methylene chloride, hexafluoroacetone and hexafluoroisopropanol.

A precipitate is thus obtained by forcing the polymer and protein/polypeptide out of the solution. The precipitation may be achieved by any technique known in the art. Suitable techniques include adding a solvent in which the polymer is not soluble or cooling the solution to achieve precipitation.

The preferred precipitation technique involves forcing the polymer out of solution using a solvent in which the protein/polypeptide is soluble but in which the polymer is not soluble. Suitable solvents include water, aqueous buffer, aqueous-alcoholic mixtures and the like. Under the proper conditions of stirring, the particle size of the precipitate can be controlled. The precipitate is then filtered and dried.

The precipitate includes both protein/polypeptide and polymer and a physical interaction is present between the protein/polypeptide and polymer. Controlled release of the protein/polypeptide in vivo is thereby achieved.

If the microsphere technique is used as depicted in Fig. 2, spherical polymer matrices or microspheres having a diameter range between about 1 to 150 microns (μm) can be prepared in narrow size ranges for

targeting to various organ or organ systems via parenteral injection or inhalation as shown in Fig. 4. A more preferred range for the spherical polymer matrices of microspheres is between about 0.5 to 70 microns.

The microspheres may be prepared by forming emulsified droplets or spheres consisting of a homogeneous mixture of polymer (or copolymer) and solvent from a solution of a preselected polymer dispersed in a continuous (non-solvent phase). Removal of the solvent from the sphere by any one or combination of (1) freeze drying, or (2) solvent extraction creates the microsphere. The protein or polypeptide may then be added.

In particular, in the microsphere method, the desired polymer or copolymer and the protein or polypeptide and other agents(s) are dissolved separately in a suitable solvent. The polymer and polypeptide solution are mixed together to provide a polymer concentration generally ranging between about 2.5 to 18% w/w and a polypeptide/polymer ratio ranging between about 1:1 to 1:100. The temperature of the resultant solution is generally controlled between about 30° to 45° C. The polypeptide-polymer solution comprising the dispersed phase is dispersed into the continuous phase containing a surface active agent at a thermostatically controlled temperature generally in the range of 10° to 20° C. Any surface active agent known in the art would be suitable in the practice of the present invention so long as it does not interfere with the activity or interaction between the polymer and protein/polypeptide. The foregoing may be accomplished by any method known in the art, in particular, by forcing the dispersed phase under pressure through a fine orifice nozzle. The continuous phase which is 5 to 20 times by weight of the dispersed phase is then agitated by a dispersator. Following the introduction of the dispersed phase, one of two recovery methods is utilized to stabilize and recover the drug-loaded microspheres for final processing.

More specifically, consistent with the freeze-dry method, following dispersion, the temperature is maintained at 10° to 20° C, preferably 15° C, for two minutes then increased to 45° to 55° C, preferably 50° C, over a three minute period. Vigorous agitation of the mixture is continued during this period. When the temperature reaches 50° C, either a refrigerant solution is circulated through the jacket from the bath or the container is immersed in dry ice-methanol and cooled to a temperature which will freeze the drug-polymer-solvent phase and not the continuous phase. The suspension or emulsion (solid dispersion phase in liquid continuous phase) is quickly transferred to precooled vials (-40° to -60° C) and cooled to -40° to -60° C in a freeze dryer, freezer or dry ice-acetone bath. The solvent in the suspended droplets (microspheres) and the continuous phase solvent are removed by freeze drying. Upon completion of the freeze dry cycle the microspheres are washed with a suitable solvent, filtered and air dried.

In the solvent extraction method of the invention, following dispersion, the temperature is maintained at 10° to 20° C, preferably 15° C, for two minutes, then increased to 45° to 55° C, preferably 50° C, over a three minute period. The dispersion is then transferred to a vessel containing a diluent solvent at room temperature or the diluent solvent is added to the dispersion. Agitation may be continued for approximately 30 minutes using an appropriate mixing technique. During the process the dispersed phase solvent is removed from the polypeptide-polymer-solvent emulsion droplets by extraction causing solidification of the droplets. The solid spheres are then removed by filtration, washed with a suitable solvent and air dried.

Solvents for the dispersed phase and the continuous phase will of course differ in order to attain phase separation and are, therefore, selected based upon the solvent requirements for each phase. More particularly, the solvent for the dispersed phase should preferably dissolve the polymer and the incorporated agent and remain in the emulsified droplets with the drug and polymer in the continuous phase until leached out by a diluent solvent or removed by vaporization or evaporation. In this way pores are optionally formed in the drug-polymer matrix. In the case of polyglycolic acid into which water soluble markers or agents are incorporated, hexafluoroacetone sesquihydrate is an appropriate solvent. Other solvents which can be used, depending upon the characteristics of the polymer and incorporated agents, include water, hexafluoro-isopropanol, methylene chloride, acetonitrile, tetrahydrofuran, hexane and benzene. Solvents for the continuous phase should not dissolve the polymer and should emulsify the dispersed phase. Suitable solvents include, but are not limited to, benzene, dioxane, acetone, methylene chloride, chloroform, carbon tetrachloride, toluene, ethyl alcohol, acetonitrile, p-xylene, tetrahydrofuran, mineral oil, glycerin and mixtures of these solvents.

A diluent (non-solvent) phase can also be employed to dilute the continuous phase following dispersion of the polymer polypeptide solution. The diluent should be miscible with the continuous phase and dispersed phase solvents but not dissolve the polymer or incorporated agent. Examples of suitable solvents include 1,4-dioxane, cyclohexanone, acetone, ethanol, isopropanol, acetonitrile, dimethylformamide, tetrahydrofuran, cyclohexanol and the like.

The concentration of polymer in the dispersed phase directly influences the porosity or "void" space in the final microsphere product as well as the shape of the microsphere. A concentration of 2.5% to 10% w/w polymer yields dimensionally suitable spherical particles. With respect to the concentration of the protein or

polypeptide, up to 50% by weight of the polymer has been achieved with consistent results.

It has been determined that certain processing parameters influence the recovery methods as well as the resultant microspheres of the present invention. Identifiable parameters include the concentration of polymer in the dispersed phase, the temperature of the dispersed phase at the time of dispersion, the concentration of surfactants in the dispersed phase as well as the ratio of incorporated agent to polymer in the dispersed phase. It will be appreciated that the concentrations, temperatures and ratios referred to hereinabove and in the Examples set forth operable ranges and that other numerical expressions may apply as different solvents, polymers, proteins, polypeptides and the like, are selected.

The present inventor wishes to emphasize that the interaction between the protein/polypeptide and the polymer of the present invention is unique. In the prior art, there was no affinity between the active drug substance and the polymer. In fact, in some instances, the affinity of the drug was much greater for the solvent in which the polymer and drug were dissolved. Thus, in the prior art systems, when the polymer was precipitated from the solution, the drug remained predominantly in the solution.

The drug delivery systems in accordance with the present invention are ideally suited for administration by the parenteral (e.g., intravenous, intraarterial, intramuscular, subcutaneous or intraocular) or inhalation routes of administration but can be used for oral and intranasal administration if such administration enhances bioavailability or reduces side effects. In particular, the macroparticulate systems in the proper size range, i.e., about 0.5μm to about 5μm, can also be administered orally for adsorption and/or pinocytosis by the mucosal cells lining the gastrointestinal tract. Such administration permits transfer of the incorporated agent intact to the systemic, lymphatic and secretory systems of the body.

It will be appreciated by those skilled in the art that the drug delivery system of the present invention may be administered alone or in admixture with appropriate pharmaceutical diluents, carriers, excipients or adjuvants suitably selected with respect to the intended route of administration and conventional pharmaceutical practices. For example, for parenteral injection, dosage unit forms may be utilized to accomplish intravenous, intramuscular or subcutaneous administration, and for such parenteral administration, suitable sterile aqueous or non-aqueous solutions or suspensions, optionally containing appropriate solutes to effectuate isotonicity, will be employed. Likewise for inhalation dosage unit forms, for administration through the mucous membranes of the nose and throat or bronchio-pulmonary tissues, suitable aerosol or spray inhalation compositions and devices will be utilized.

Consistent with other preferred embodiments of the present invention, the drug delivery system of the invention may be additionally coated or modified to advantageously influence the targeting of the release of the incorporated drug therein to preselected target cells, tissues or organs. For example, the drug delivery microspheres may be coated with various agents, e.g., polymers, proteins, surfactants, antibodies or receptor site specific drugs which may be the same or different from those incorporated in the microsphere whereby the release of the incorporated drug is concentrated at the targeted system. Additionally, the coatings can be pH sensitive so as to effect protection following oral administration and transit through the stomach.

In order further to illustrate the present invention and the advantages thereof, the following specific examples are given, it being understood that same are intended only as illustrative, not limitative.

EXAMPLE 1

Molecular Interaction of Salmon Calcitonin with Polyglycolic Acid

The present example was intended to quantify chemical and/or physical association between salmon calcitonin and polyglycolic acid (PGA) having a molecular weight of 40,000 Daltons.

Approximately five mg of calcitonin were quantitatively weighed and placed in each of a series of 5 ml volumetric flasks. Hexafluoroacetone sesquihydrate (HFA) was added dropwise until the calcitonin dissolved completely. A 5% PGA in HFA solution was quantitatively added dropwise to each flask to provide a mass of PGA covering the range of 0 to 26.3 mg. The flasks were agitated for 5 minutes to mix the solution. Each flask was then filled to the 5 ml mark with phosphate buffer (pH 7.3). The addition of buffer precipitated the PGA plus any calcitonin that had bound with the polymer. The resulting mixture was centrifuged and the supernatant was analyzed spectrophotometrically for salmon calcitonin content. 26 mg of PGA (40,000 Mw) removed approximately 4.1 mg (83%) of salmon calcitonin. The results are set forth in Table I.

TABLE I

PGA MASS (mg)	SUPERNATANT ABS (275nm)	CALCITONIN CONC.(mg/ml)	CALCITONIN REMOVED (mg)	%CALCITONIN REMOVED
0.00	0.390	1.020	0.099	1.90
5.73	0.342	0.895	0.125	2.71
7.81	0.285	0.746	1.07	22.3
7.89	0.356	0.932	0.340	6.80
11.8	0.213	0.558	1.71	38.0
13.2	0.257	0.673	1.64	32.7
14.0	0.220	0.576	2.02	41.2
15.8	0.176	0.461	2.69	53.7
18.4	0.086	0.226	3.87	77.4
10.4	0.165	0.432	2.54	54.0
21.0	0.068	0.175	4.10	82.1
22.3	0.161	0.422	2.95	58.6
23.7	0.105	0.276	3.62	72.4
26.3	0.066	0.174	4.13	82.6

EXAMPLE 2

Molecular Interaction of Salmon Calcitonin with Poly(Glycolic-Co-Lactic Acid)

The procedure of preparing the poly(glycolic-Co-lactic acid) (PGL) having a molecular weight of 50,000 microspheres was similar to that used for PGA, except the hexafluoro-2-propanol was substituted for HFA. Above 8 mg of PGL over 80% of salmon calcitonin was removed. The results are set forth in Table II.

TABLE II

PGL MASS (mg)	SUPERNATANT ABS (275nm)	CALCITONIN CONC. (mg/ml)	CALCITONIN REMOVED (mg)	%CALCITONIN REMOVED
0.00	0.360	0.941	0.591	11.1
2.64	0.306	0.801	0.995	19.9
4.13	0.210	0.550	2.15	43.9
5.52	0.191	0.501	2.69	51.8
6.51	0.147	0.386	2.87	59.8
8.45	0.065	0.172	3.64	80.8
11.53	0.047	0.125	4.37	87.5
13.57	0.027	0.0725	4.44	92.5
20.97	0.034	0.0908	4.95	91.6

EXAMPLE 3

Molecular Interaction of Salmon Calcitonin with Polylactic Acid

The procedure for preparing the polylactic acid (PLA), dl-type having a molecular weight of 50,000, was similar to that used for PGA, except that methylene chloride was substituted for HFA and the calcitonin was suspended rather than dissolved in the methylene chloride. In addition, since methylene chloride and buffer are not miscible, salmon calcitonin was extracted from methylene chloride in buffer. The aqueous phase was separated, centrifuged and the supernatant was analyzed spectrophotometrically for salmon calcitonin. The results are set forth in Table III.

TABLE III

PLA MASS (mg)	SUPERNATANT ABS (275nm)	CALCITONIN CONC. (mg/ml)	CALCITONIN REMOVED (mg)	%CALCITONIN REMOVED
0.00	0.401	1.05	0.055	1.03
2.68	0.226	0.592	2.04	40.8
4.81	0.186	0.488	2.36	47.2
10.28	0.147	0.386	2.77	55.4
14.95	0.105	0.276	3.62	72.4
20.40	0.159	0.417	3.02	60.3

EXAMPLE 4

Molecular Interaction of Salmon Calcitonin with Pure Polymer

Approximately 100 mg of PGA (Mw 40,000) was placed in a vial. Ten ml of a 1 mg/ml calcitonin in phosphate buffer (pH 7.3) were added to the vial. The polymer was suspended in the calcitonin solution by placing the vial in an ultrasonic bath for 10 minutes. The suspension was then centrifuged and the supernatant was analyzed spectrophotometrically. This procedure was repeated for PGA (MW 100,000), PGL and PLA (dl-type) polymers, with the revision that all quantitative amounts were halved for the PGL and PLA trials. The results are set forth in Table IV.

TABLE IV

POLYMER	MASS(mg)	SUPERNATANT ABS (275nm)	CALCITONIN REMOVED(mg)	mg sCT per mg POLYMER REMOVED
PGA (40,000)	99.1	0.4022	0.723	0.723/99.1
PGA (100,000)	99.3	0.3501	1.143	1.143/99.3
PGL (50,000)	50.0	0.4144	0.457	0.457/50.3
PLA (dl-type) (50,000)	50.0	0.2474	2.772	2.772/50.0

The pure polymers showed a binding affinity, up to 5.5%, which was less than the molecular interaction during the precipitation process. PLA (dl-type) showed the highest affinity to bind with salmon calcitonin when suspended in a 1 mg/ml solution.

Since the PGA system was the polymer of choice for preparing microspheres by the freeze drying technique, some efforts were made to determine the nature of the association between drug and polymer. Utilizing differential scanning calorimetry there was some shifts in the melting points of salmon calcitonin and PGA when these agents were combined in microspheres. Changes were also observed in the I.R. and Raman spectra. All of these suggest an association but do not conclusively point to the precise nature of the interaction. However, Fourier Transfer Raman spectrometry did not show any discernible differences. This suggests that the interaction is not chemical or covalent in nature.

EXAMPLE 5

Preparation of a Salmon Calcitonin-PGA Precipitated System

1. Precipitation with Water

49.3 mg of salmon calcitonin was placed in a vial and dissolved with 0.35 ml HFA sesquihydrate. A 4.5

g of a 10% PGA-HFA solution containing 450 mg of PGA was added dropwise to the solution while stirring with a magnetic stirring bar. The mixture was agitated for 5 additional minutes. A pH 7.3 phosphate buffer was then added to the mixture to precipitate the polymer. Turbidity suggested the precipitation of the polymer. The mixture was agitated two more minutes using a vortex mixer and then centrifuged for 10 minutes at 3000 rpm. The supernatant was saved for analysis and the precipitate dried in a low pressure chamber for a few hours. The salmon calcitonin of the content of the supernatant was analyzed spectrophotometrically and the amount of the active agent removed by the polymer was calculated. Loading was between 6.0 to 8.0% by weight of polymer.

2. Precipitation with Ethyl Alcohol

The buffer was replaced by ethanol to precipitate the polymer to attempt to improve the yield. The total solid input (polymer + salmon calcitonin) was 502 mg in this preparation. A loading of 6.4 to 8.0% was used.

EXAMPLE 6

Characterization of Salmon Calcitonin-PGA Microspheres and Precipitate

1. Blank Microspheres

a) Drug Load

Microspheres were prepared as described in Example 5 using 100,000 molecular weight polyglycolic acid. Because of the association tendency of salmon calcitonin with polyglycolic acid, it was not possible to use the precipitation technique to determine actual drug load. The 30 minute extraction technique in buffer was more indicative of actual salmon calcitonin content. By the extraction method and HPLC analysis, a drug content of 8.21 % w/w was calculated as 82% incorporation efficiency.

b) In Vitro Release of Salmon Calcitonin

Twenty mg of PGA-salmon calcitonin microspheres were loaded into a test tube. Ten ml of a 0.1M phosphate buffer, pH 7.4, containing EDTA were added and the tubes transferred to a 37° C water bath. Results of this study are shown in Fig. 5. Nineteen μg salmon calcitonin/mg microspheres were released initially as a burst. This initial release was followed by a fast release of 50% of the total drug in less than two hours. At this point, a slow release followed and 22 μg salmon calcitonin/mg microspheres (22% of total drug) was released in the following 29 hours. The data from this study suggested that salmon calcitonin remains stable in the phosphate buffer for about 35 hours.

2. Precipitate

a) Size Distribution

HIAC/ROYCO counter-size analyzer was used to analyze the drug loaded precipitate for size distribution. As shown in Fig. 2, D_{50}, the number median diameter, was approximately 2.8 μm and 99% of the particles were in the range of 2-10 μm. The geometric standard deviation, σ_g = 1.83 is indicative of a fair monodispersity. MMD, mass median diameter, was calculated to be 4.39 μm.

b) In Vitro Release of Salmon Calcitonin from Precipitate

Two 18 mg samples of salmon calcitonin-PGA precipitate were quantitatively transferred to test tubes. Ten ml of a 0.1M phosphate buffer, pH 7.4, containing EDTA were added to the samples and placed in a 37° C shaker bath. Samples were withdrawn at predetermined time intervals and analyzed for drug content. As shown in Fig. 6, the initial release was rapid. A release of 22% occurred at zero time, followed by another 21% of the total drug released in less than 2 hours. This was followed with only negligible release for the next 30 hours of about 0.1% per hour. A significant amount of salmon calcitonin appears to be still within the matrix or bound to the polymer.

EXAMPLE 7

In Vivo Assessment of Salmon Calcitonin Sustained Release From Biodegradable Microspheres

Biodegradable microspheres containing salmon calcitonin were prepared with polyglycolic acid, 40,000D by a freeze-drying technique as depicated in Fig. 2. Salmon calcitonin microspheres with different salmon calcitonin contents were characterized for particle size, porosity, specific surface area and in- vitro release. The sustained hypocalcemic effect was assessed by subcutaneous injection in male Wistar rats, followed by blood sampling via femoral artery catheter at defined time intervals and assaying for serum calcium concentrations. Drug loads of 0.3, 4.5 and 7.5% were evaluated and a level of 0.3% was found to be effective in producing a sustained hypocalcemic effect. With this drug load, salmon calcitonin microspheres containing 40, 120 and 360 mU of salmon calcitonin per 100g body weight were administered as illustrated in Fig. 7. The hypocalcemic effect was sustained for a 24 hour period with the salmon calcitonin microspheres as compared to 2 to 3 hours with free salmon calcitonin. Additionally, salmon calcitonin blood levels were sustained at concentrations higher than baseline for a period of five days.

From the foregoing description, one of ordinary skill in the art can easily ascertain the essential characteristics of the instant invention, and without departing from the spirit and scope thereof, can make various changes and/or modifications of the invention to adapt it to various usages and conditions. As such, these changes and/or modifications are properly, equitably and intended to be within the full range of equivalence of the following claims.

Claims

1. A drug delivery system for controlled release of a protein or polypeptide, said drug delivery system comprising a hydrophobic biodegradable polymer and a protein or polypeptide, wherein a physical interaction is present between said polymer and protein or polypeptide such that protection and controlled release of the protein or polypeptide is achieved in vivo.

2. The drug delivery system as claimed in claim 1, wherein said polymer is selected from the group consisting of polyester, polyorthoester and polyanhydride.

3. The drug delivery system as claimed in claim 2, wherein said polyester is selected from the group consisting of polyglycolic acid, polylactic acid and copolymers of glycolide and L-lactide.

4. The drug delivery system as claimed in claim 1, wherein said protein or polypeptide is selected from the group consisting of calcitonin, insulin, angiotensin, vasopressin, desmopressin, luteinizing hormone-releasing hormone, somatostatin, glucagon, somatomedin, oxytocin, gastrin, secretin, human atrial natriuretic polypeptide, adrenocorticotropic hormone, melanocyte stimulating hormone, beta-endorphin, muramyl dipeptide, enkephalin, neurotensin, bombesin, VIP, CCK-8, parathyroid hormone, calcitonin gene related peptide, endothelin, thyroid releasing hormone, growth hormone and lymphokines.

5. The drug delivery system as claimed in claim 4, wherein said protein or polypeptide is calcitonin.

6. A drug delivery system for controlled release of protein or polypeptide, said drug delivery system comprising a hydrophobic biodegradable polymer selected from polyglycolic acid, polylactic acid and copolymers of glycolide and L-lactide, and calcitonin, wherein a physical interaction is present between said polymer and calcitonin such that controlled release of the calcitonin is achieved in vivo.

7. An oral drug delivery system for controlled release of a protein or polypeptide, said oral drug delivery system comprising a hydrophobic biodegradable polymer and a protein or polypeptide, wherein a physical interaction is present between said polymer and proteins or polypeptide whereby protection and controlled release of the protein or polypeptide is achieved in vivo and the intact drug delivery system is adsorbed from the gastrointestinal tract into the mucosal lining.

8. The drug delivery system as claimed in claim 7, wherein said polymer is selected from the group consisting of polyester, polyorthoester and polyanhydride.

9. The drug delivery system as claimed in claim 8, wherein said polyester is selected from the group

consisting of polyglycolic acid, polylactic acid and copolymers of glycolide and L-lactide.

10. The drug delivery system as claimed in claim 7, wherein said protein or polypeptide is selected from the group consisting of calcitonin, insulin, angiotensin, vasopressin, desmopressin, luteinizing hormone-releasing hormone, somatostatin, glucagon, somatomedin, oxytocin, gastrin, secretin, human atrial natriuretic polypeptide, adrenocorticotropic hormone, melanocyte stimulating hormone, beta-endorphin, muramyl dipeptide, enkephalin, neurotensin, bombesin, VIP, CCK-8, parathyroid hormone, calcitonin gene related peptide, endothelin, thyroid releasing hormone, growth hormone and lymphokines.

11. The drug delivery system as claimed in claim 10, wherein said protein or polypeptide is calcitonin.

12. A process for preparing a drug delivery system for controlled release of a protein or polypeptide, said process comprising:

a) dissolving a protein or polypeptide and a hydrophobic biodegradable polymer in a solvent, and

b) forming a precipitate containing the protein or polypeptide and polymer formed as a result of the interaction between the protein or polypeptide and the polymer.

13. The process as claimed in claim 12, wherein the precipitate is formed by cooling the solution comprising the solvent, protein or polypeptide and polymer.

14. The process as claimed in claim 12, wherein the precipitate is reduced in size.

15. A process for preparing a drug delivery system for controlled release of a protein or polypeptide, said process comprising:

a) dissolving a protein or polypeptide and a hydrophobic biodegradable polymer in a first solvent, and

b) adding a second solvent in which the protein or polypeptide is soluble but in which the hydrophobic biodegradable polymer is not soluble in order to form a precipitate containing the protein or polypeptide and polymer formed as a result of the interaction between the protein or polypeptide and the polymer.

16. The process as claimed in claim 15, wherein said polymer is selected from the group consisting of polyester, polyorthoester and polyanhydride.

17. The process as claimed in claim 16, wherein said polyester is selected from the group consisting of polyglycolic acid, polylactic acid and copolymers of glycolide and L-lactide.

18. The process as claimed in claim 15, wherein said protein or polypeptide is selected from the group consisting of calcitonin, insulin, angiotensin, vasopressin, desmopressin, luteinizing hormone-releasing hormone, somatostatin, glucagon, somatomedin, oxytocin, gastrin, secretin, human atrial natriuretic polypeptide, adrenocorticotropic hormone, melanocyte stimulating hormone, beta-endorphin, muramyl dipeptide, enkephalin, neurotensin, bombesin, VIP, CCK-8, parathyroid hormone, calcitonin gene related peptide, endothelin, thyroid releasing hormone, growth hormone and lymphokines.

19. The process as claimed in claim 18, wherein said protein or polypeptide is calcitonin.

20. The process as claimed in claim 15, wherein said first solvent is selected from the group consisting of methylene chloride, hexafluoroacetone, hexafluoroisopropanol, acetonitrile, hexane and cyclohexane.

21. The process as claimed in claim 15, wherein said second solvent is selected from the group consisting of water, aqueous buffer and aqueous-alcoholic mixture.

22. The process as claimed in claim 15, wherein the precipitate is reduced in size.

23. A process for preparing a drug delivery system for controlled release of a protein or polypeptide, said process comprising forming a microsphere containing the protein or polypeptide and the hydrophobic biodegradable polymer whereby a physical interaction exists between the protein or polypeptide and the hydrophobic biodegradable polymer.

24. A process for preparing a drug delivery system for controlled release of a protein or polypeptide, said process comprising:
a) dissolving a protein or polypeptide and hydrophobic biodegradable polymer in a solvent to form a first phase;
b) dispersing said first phase in a continuous solvent second phase to obtain a suspension, and
c) removing said solvent from said suspension by freeze-drying a solvent extraction to obtain a precipitate containing the protein or polypeptide and polymer formed as a result of the interaction between the protein or polypeptide and the polymer.

25. The process as claimed in claim 24, wherein said polymer is selected from the group consisting of polyester, polyorthoester and polyanhydride.

26. The process as claimed in claim 25, wherein said polyester is selected from the group consisting of polyglycolic acid, polylactic acid and copolymers of glycolide and L-lactide.

27. The process as claimed in claim 24, wherein said protein or polypeptide is selected from the group consisting of calcitonin, insulin, angiotensin, vasopressin, desmopressin, luteinizing hormone-releasing hormone, somatostatin, glucagon, somatomedin, oxytocin, gastrin, secretin, human atrial natriuretic polypeptide, adrenocorticotropic hormone, melanocyte stimulating hormone, beta-endorphin, muramyl dipeptide, enkephalin, neurotensin, bombesin, VIP, CCK-8, parathyroid hormone, calcitonin gene related peptide, endothelin, thyroid releasing hormone, growth hormone and lymphokines.

28. The process as claimed in claim 27, wherein said protein or polypeptide is calcitonin.

29. The process as claimed in claim 24, wherein the precipitate is reduced in size.

Claims for the following Contracting States: ES GR

1. A process for preparing a drug delivery system for controlled release of a protein or polypeptide, said process comprising:
a) dissolving a protein or polypeptide and a hydrophobic biodegradable polymer in a solvent, and
b) forming a precipitate containing the protein or polypeptide and polymer formed as a result of the interaction between the protein or polypeptide and the polymer.

2. The process as claimed in claim 1, wherein the precipitate is formed by cooling the solution comprising the solvent, protein or polypeptide and polymer.

3. The process as claimed in claim 1, wherein the precipitate is reduced in size.

4. A process for preparing a drug delivery system for controlled release of a protein or polypeptide, said process comprising:
a) dissolving a protein or polypeptide and a hydrophobic biodegradable polymer in a first solvent, and
b) adding a second solvent in which the protein or polypeptide is soluble but in which the hydrophobic biodegradable polymer is not soluble in order to form a precipitate containing the protein or polypeptide and polymer formed as a result of the interaction between the protein or polypeptide and the polymer.

5. The process as claimed in claim 4, wherein said polymer is selected from the group consisting of polyester, polyorthoester and polyanhydride.

6. The process as claimed in claim 5, wherein said polyester is selected from the group consisting of polyglycolic acid, polylactic acid and copolymers of glycolide and L-lactide.

7. The process as claimed in claim 4, wherein said protein or polypeptide is selected from the group consisting of calcitonin, insulin, angiotensin, vasopressin, desmopressin, luteinizing hormone-releasing hormone, somatostatin, glucagon, somatomedin, oxytocin, gastrin, secretin, human atrial natriuretic polypeptide, adrenocorticotropic hormone, melanocyte stimulating hormone, beta-endorphin, muramyl

dipeptide, enkephalin, neurotensin, bombesin, VIP, CCK-8, parathyroid hormone, calcitonin gene related peptide, endothelin, thyroid releasing hormone, growth hormone and lymphokines.

8. The process as claimed in claim 7, wherein said protein or polypeptide is calcitonin.

9. The process as claimed in claim 4, wherein said first solvent is selected from the group consisting of methylene chloride, hexafluoroacetone, hexafluoroisopropanol, acetonitrile, hexane and cyclohexane.

10. The process as claimed in claim 4, wherein said second solvent is selected from the group consisting of water, aqueous buffer and aqueous-alcoholic mixture.

11. The process as claimed in claim 4, wherein the precipitate is reduced in size.

12. A process for preparing a drug delivery system for controlled release of a protein or polypeptide, said process comprising forming a microsphere containing the protein or polypeptide and the hydrophobic biodegradable polymer whereby a physical interaction exists between the protein or polypeptide and the hydrophobic biodegradable polymer.

13. A process for preparing a drug delivery system for controlled release of a protein or polypeptide, said process comprising:

a) dissolving a protein or polypeptide and hydrophobic biodegradable polymer in a solvent to form a first phase;

b) dispersing said first phase in a continuous solvent second phase to obtain a suspension, and

c) removing said solvent from said suspension by freeze-drying a solvent extraction to obtain a precipitate containing the protein or polypeptide and polymer formed as a result of the interaction between the protein or polypeptide and the polymer.

14. The process as claimed in claim 13, wherein said polymer is selected from the group consisting of polyester, polyorthoester and polyanhydride.

15. The process as claimed in claim 14, wherein said polyester is selected from the group consisting of polyglycolic acid, polylactic acid and copolymers of glycolide and L-lactide.

16. The process as claimed in claim 13, wherein said protein or polypeptide is selected from the group consisting of calcitonin, insulin, angiotensin, vasopressin, desmopressin, luteinizing hormone-releasing hormone, somatostatin, glucagon, somatomedin, oxytocin, gastrin, secretin, human atrial natriuretic polypeptide, adrenocorticotropic hormone, melanocyte stimulating hormone, beta-endorphin, muramyl dipeptide, enkephalin, neurotensin, bombesin, VIP, CCK-8, parathyroid hormone, calcitonin gene related peptide, endothelin, thyroid releasing hormone, growth hormone and lymphokines.

17. The process as claimed in claim 16, wherein said protein or polypeptide is calcitonin.

18. The process as claimed in claim 13, wherein the precipitate is reduced in size.

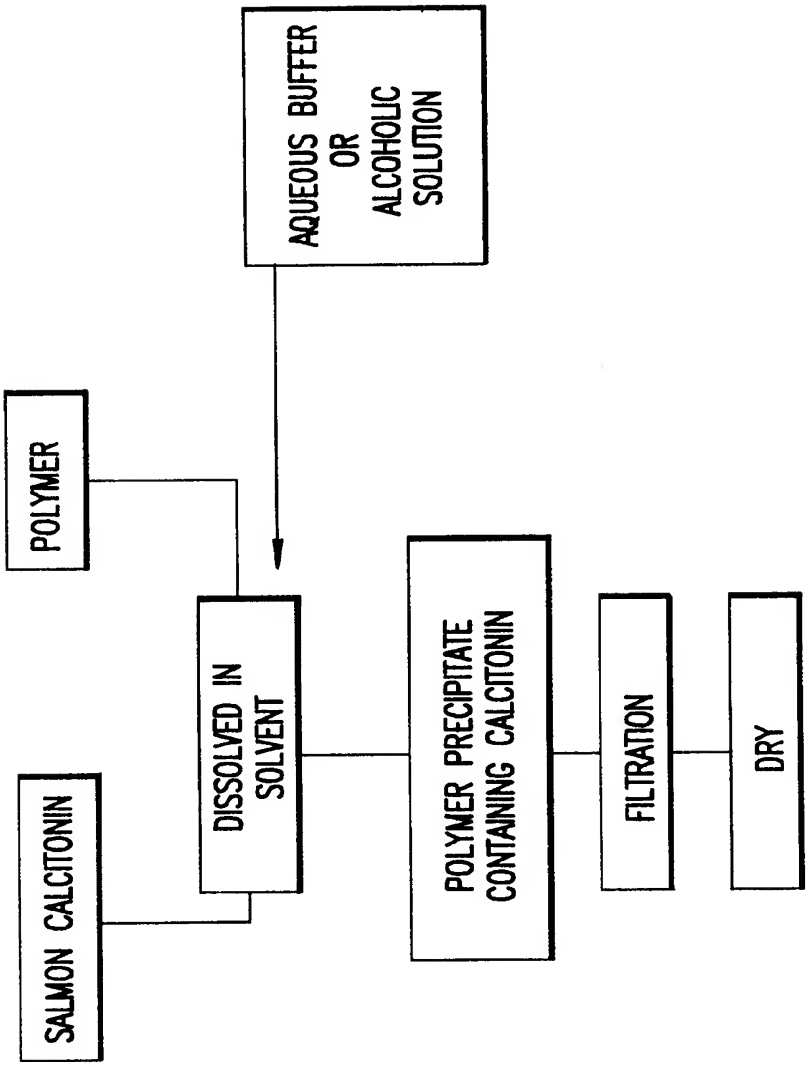

AQUEOUS BUFFER OR ALCOHOLIC SOLUTION
POLYMER
SALMON CALCITONIN
DISSOLVED IN SOLVENT
POLYMER PRECIPITATE CONTAINING CALCITONIN
FILTRATION
DRY

FIG. 1

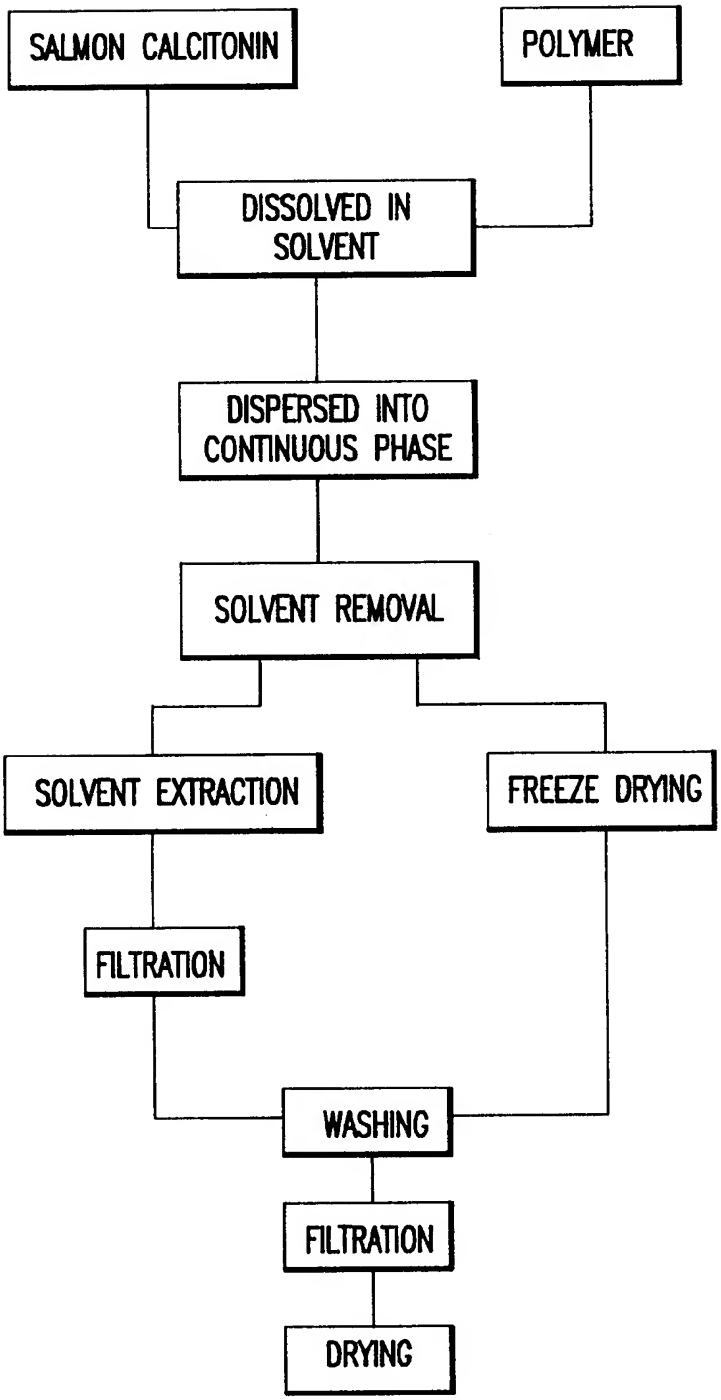
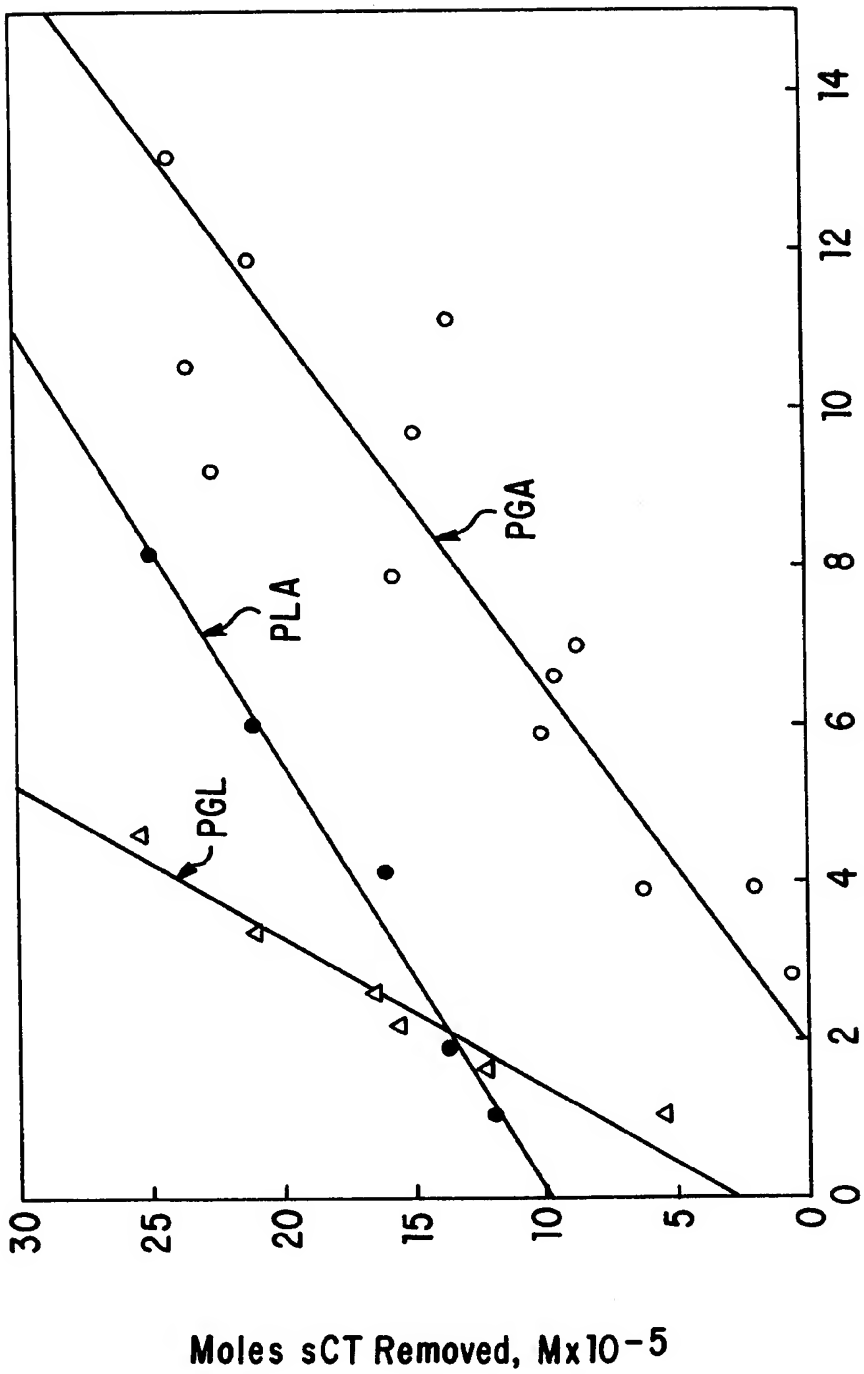

SALMON CALCITONIN
POLYMER
DISSOLVED IN SOLVENT
DISPERSED INTO CONTINUOUS PHASE
SOLVENT REMOVAL
SOLVENT EXTRACTION
FREEZE DRYING
FILTRATION
WASHING
FILTRATION
DRYING

FIG.2


PGA
PLA
PGL
0
2
4
6
8
10
12
14
Polymer Concentration, Mx10-5
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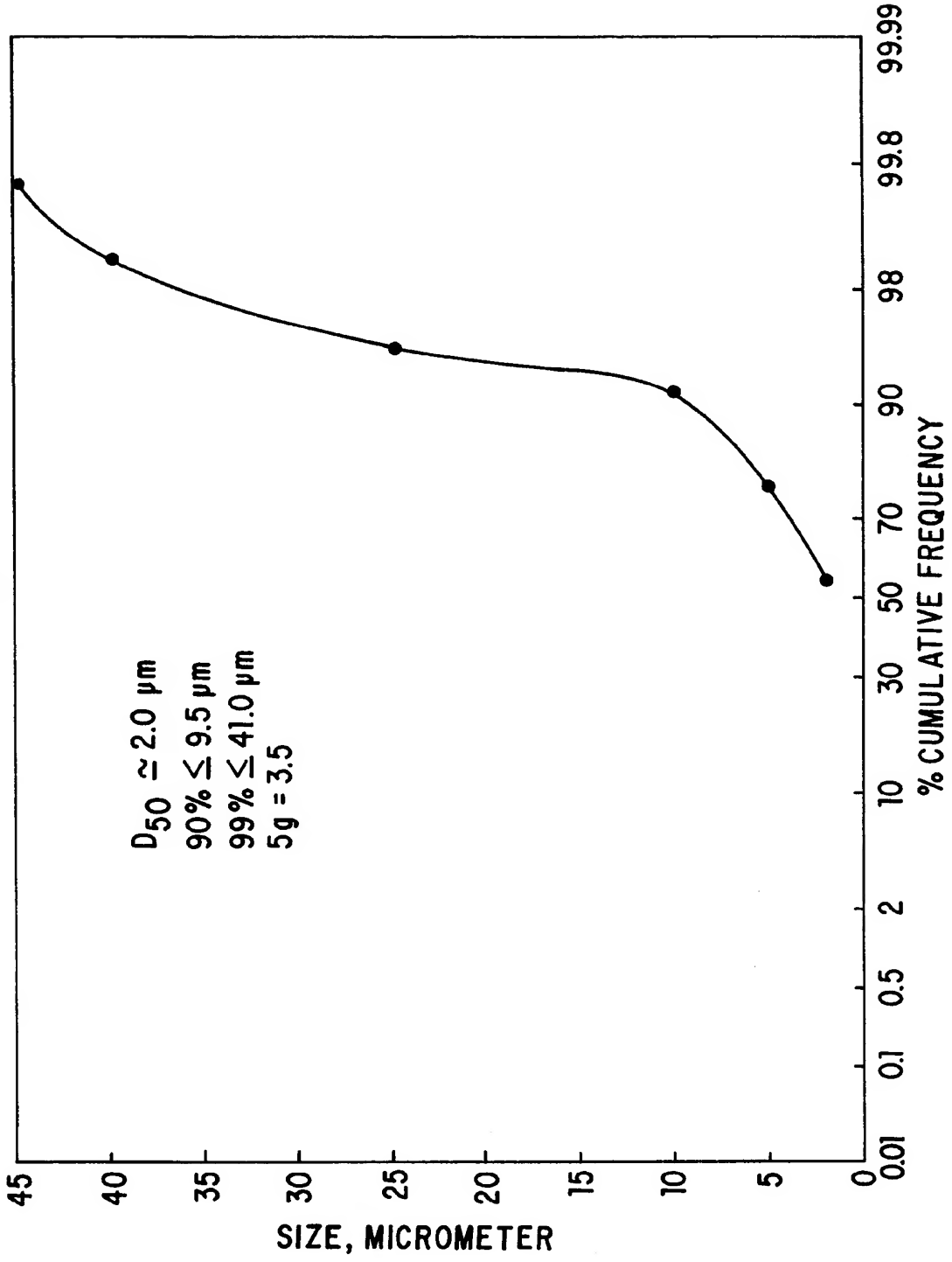
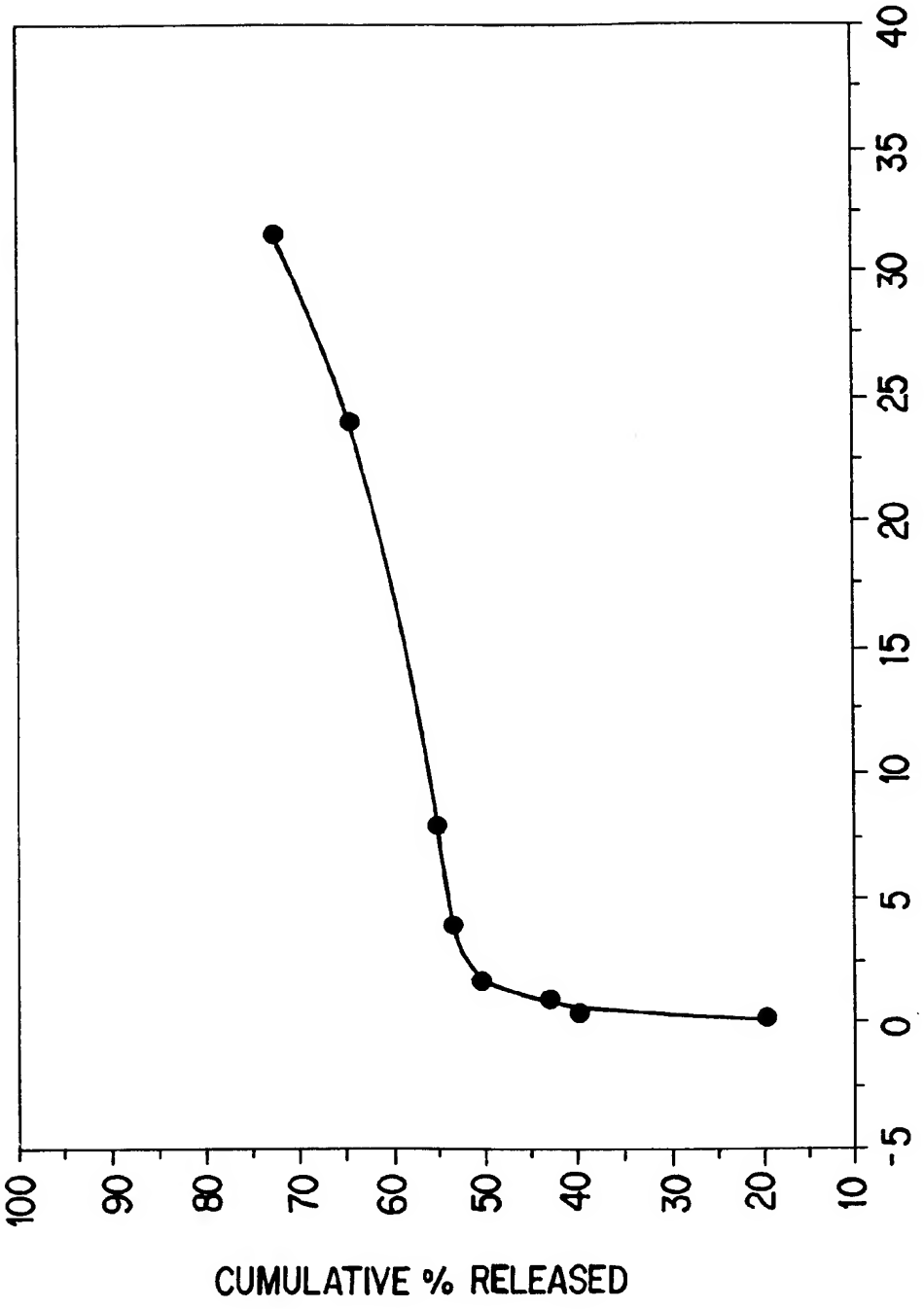
10
15
20
25
30
Moles sCT Removed, Mx10-5

FIG. 3

FIG. 4


$D_{50} \simeq 2.0$ µm
90% ≤ 9.5 µm
99% ≤ 41.0 µm
Sg = 3.5
SIZE, MICROMETER
45
40
35
30
25
20
15
10
5
0
0.01
0.1
0.5
2
10
30
50
70
90
98
99.8
99.99
% CUMULATIVE FREQUENCY


100
90
80
70
60
50
40
30
20
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10
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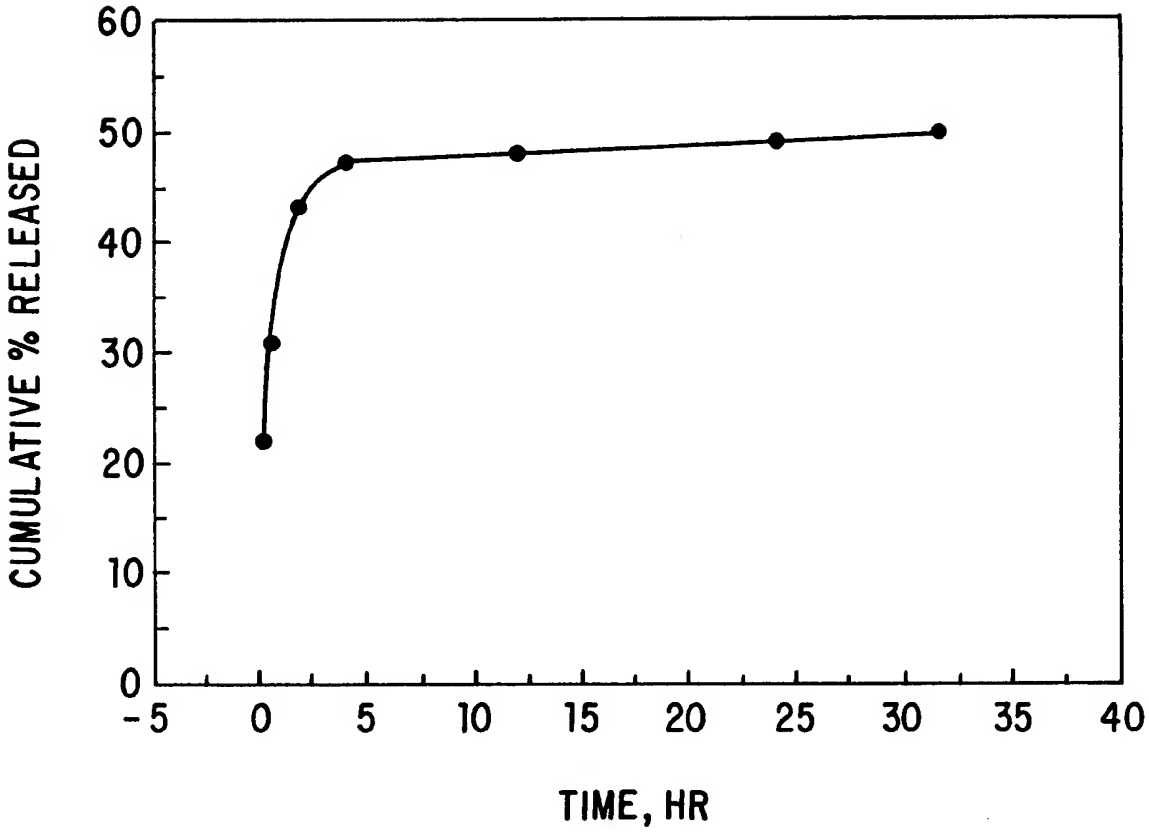
20
25
30
35
40
TIME, HR.

FIG. 5


CUMULATIVE % RELEASED
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40
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0
-5
0
5
10
15
20
25
30
35
40
TIME, HR

FIG. 6

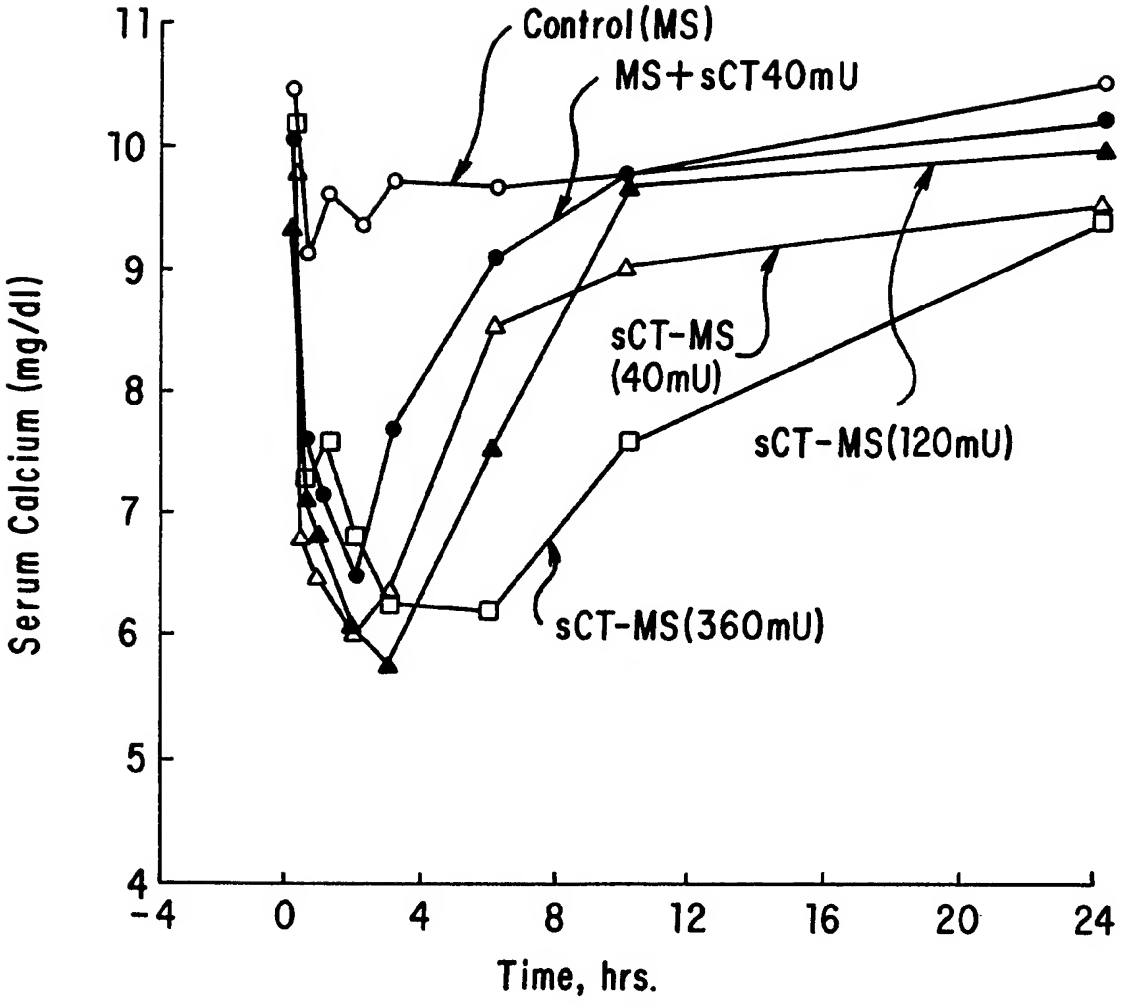
Control(MS)
MS+sCT40mU
sCT-MS
(40mU)
sCT-MS(120mU)
sCT-MS(360mU)
Serum Calcium (mg/dl)
Time, hrs.
11
10
9
8
7
6
5
4
-4
0
4
8
12
16
20
24

FIG. 7

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(54) **Drug delivery system involving interaction between protein or polypeptide and hydrophobic biodegradable polymer.**

(57) A drug delivery system for controlled release of a protein or polypeptide comprising a hydrophobic biodegradable polymer and a protein or polypeptide. A physical interaction is present between the polymer and the protein or polypeptide, thus, allowing protection and controlled release of the protein or polypeptide in-vivo. The drug delivery system may be prepared by a polymer precipitation technique or a microsphere technique.

EP 0 467 389 A3

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 91 11 2118

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
X,P	US-A-5 008 116 (F. CAHN) * claims 1-11 *	1-3,7-9	A61K9/52 A61K9/16 A61K37/02
X,P	WO-A-9 106 286 (ENZYTECH INC.) * page 13, line 3 - line 19; claims 1-45; example 1 *	1,4,7, 10,12	
X	WO-A-8 809 664 (MASSACHUSETTS INSTITUTE OF TECHNOLOGY) * page 5, line 16 - page 6, line 20; claims 1-14 *	1,4,7,10	
X	EP-A-0 058 481 (IMPERIAL CHEMICAL INDUSTRIES PLC) * page 5, line 25 - line 30 * * page 6, line 25 - page 8, line 31 * * claims 1-23; examples 31-32,34 *	1-13, 24-28	
Y	EP-A-0 330 180 (BIOMATERIALS UNIVERSE INC.) * page 3, line 46 - line 56; claims 1-9 *	1-29	TECHNICAL FIELDS SEARCHED (Int. Cl.5)
Y,D	US-A-4 818 542 (P. P. DELUCA ET AL) * column 2, line 51 - line 68; claims 1-25 *	1-29	A61K C07K
A D	EP-A-0 245 820 (THE UNIVERSITY OF KENTUCKY RESEARCH FOUNDATION) * abstract * & US-A-4 741 872	1-29	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
BERLIN	30 NOVEMBER 1993	SIATOU E.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date.
D : document cited in the application
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(54) Physiologically active substance-prolonged releasing-type pharmaceutical preparation.

(57) A prolonged releasing pharmaceutical preparation is provided carrying a physiologically active substance, particularly, calcitonin gene-related peptide (CGRP) or a maxadilan (MAX). This pharmaceutical preparation can attain the expected effects by incorpolating the physiologically active substance into a combination, as carriers for the physiologically active substance, of a cellulosic polymer and at least one auxiliary component selected from the group consisting of fats and oils, waxes, fatty acids, saccharides and polyacrylate ester derivatives. The pharmaceutical preparation can conveniently be used, in living bodies, particularly as an intrathecal implantation-type preparation.

EP 0 645 136 A2

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to a pharmaceutical preparation capable of prolonged releasing a physiologically active substance in a controlled state. The pharmaceutical preparation is, particularly, directed to physiologically active peptides such as calcitonin gene-related peptide (CGRP) and maxadilans (MAXs). The pharmaceutical preparation of the invention is useful as an intracorporeal implantation-type, particularly intrathecal implantation-type physiologically active substance-prolonged releasing preparation.

2. Background of the Invention

Prolonged releasing (hereinafter, the same as gradually or delayed releasing) preparations, wherein when the drugs or physiologically active substances are administered into living bodies, elution of the drugs in the living bodies is controlled and their absorption is adjusted, have been investigated from long ago. For example, a method which comprises coating drugs with various coats, a method which comprises incorporating drugs into matrices of waxes or macromolecules, etc. have been known.

However, when intrathecal diseases are treated, in the case of intravenous administration of physiologically active substances, migration of these physiologically active substances into the brains is prevented by the blood brain barriers. As a method for direct administration of a physiologically active substance into brains, there is a method which comprises seating a catheter at the time of the operation and gradually supplying the drug into the brain, but since the apparatus is expensive and in addition there is a large danger of infection, it is hard to say that the method is a reliable method. For example, in delayed cerebral vasospasm occurring after subarachnoid hemorrhage, the pathosis is retardingly manifested and moreover is lasting, and thus a method which comprises inserting a catheter for administration of drugs and a method which comprises continuously administering a drug into the vein are used. However, it is the present state of things that a method for obtaining sure therapeutic effects has not yet been developed.

When one's eyes are turned to physiologically active peptides, particularly calcitonin gene-related peptide (CGRP) and maxadilans (MAXs) about which the present inventors have contemplated development of pharmaceutical preparations effective for various diseases, prolonged releasing pharmaceutical preparations for active substances specifically effective in relation to targeted diseases have not been proposed. When CGRP and maxadilans are specifically taken up, these are extremely interesting as proteins capable of inducing vasodilative and temporary immune suppression in mammals, as disclosed in E. A. Lerner et al., International Publication No. WO 91/00293, but after the report of Lerner et al., prolonged releasing pharmaceutical preparations therefor have not been proposed.

In this connection, the "maxadilans" were described in the above publication as proteins derived from the salivary gland lysate of a sand fly Lutzomyia longipalpis, and, thereafter, named "maxadilans" by them (for example, J. Biol. Chem., vol. 267, 1062-1066, 1992). Lerner et al. exhibit, in the just above literature, through expression of recombinant maxadilans, that the analogues of maxadilan disclosed in WO 91/00293 also have a vasodilative activity. Other maxadilan analogues having a vasodilative activity are disclosed in M. Ohnuma, E. A. Lerner et al., Peptide Chemistry 1993: Y. Okada (Ed.), 145-148.

As an interesting report from the aspect of the pharmacological actions of CGRP, H. Shimizu et al., Nou Shinkei Geka (Neurosurgery), 22(2): 131-139, 1994 discloses that when subarachnoid hemorrhage models of rabbits are used, and portions of an aseptic solution of CGRP (human alpha CGRP: Bachem Feinchemikalien, AG, Budendorf, Switzerland) are injected into the cisterna magnas of the animals, respectively, dilation effects on the contracted blood vessels are obtained.

However, this method is not always satisfactory in the effects of prophylaxis or treatment of cerebral vasospasm where the pathosis is retardingly manifested as stated above, and moreover, it is necessary to continue to strictly monitor the injection of the aqueous solution of the physiologically active substance into the cisterna magna, since said injection is very dangerous, during the injection operation.

Thus, the object of this invention lies in providing a pharmaceutical preparation effective for prophylaxis or treatment of, especially cerebral vasospasm, and a method therefor.

SUMMARY OF THE INVENTION

The present inventors, in order to accomplish the above object, have investigated above combinations of the contemplated physiologically active substances with various carriers, and have found, unexpectedly, that when a cellulosic polymer-based carrier is used, the physiologically active substance, particularly the

physiologically active peptide exhibits controlled releasability. As a further result of this research, they also found that MAXs themselves are effective for prophylaxis and treatment of cerebral vasospasm, irrespective of their dosage forms.

Another important finding found by the present inventors is that a method to implant a prolonged releasing pharmaceutical preparation containing a compound having a vasodilative action into the brain, which method has hitherto not been tried at all as a method to present or treat cerebral vasospasm, and has not been disclosed nor suggested in scientific literatures, etc., is extremely effective for prophylaxis or treatment of the disease.

Thus, this invention is directed to a pharmaceutical preparation carrying an effective amount of a physiologically active substance and capable of prolonged releasing the physiologically active substance, wherein the carrier of the physiologically active substance comprises a combination of a cellulosic polymer and at least one auxiliary component selected from fats and oils, waxes, fatty acids, saccharides and polyacrylate ester derivatives.

As a more specific embodiment, this invention is directed to a pharmaceutical preparation carrying an effective amount of a physiologically active substance and capable of prolonged releasing the physiologically active substance, wherein

the physiologically active substance is one or more selected from the group consisting of CGRP and MAXs, and

the carrier of the physiologically active substance comprises 10 to 90 % by weight of a cellulose ether derivative, 1 to 30 % by weight of a fat or oil or a wax, and 1 to 30 % by weight of a fatty acid, based on the total weight of the pharmaceutical preparation; or

the carrier of the physiologically active substance comprises 10 to 90 % by weight of a cellulose ether derivative and 1 to 40 % by weight of a saccharide, based on the total weight of the pharmaceutical preparation; or

the carrier of the physiologically active substance is a combination of 10 to 90 % by weight of crystalline cellulose and 0.01 to 10 % by weight of a polyacrylate ester derivative with at least one or more selected from the group consisting of 1 to 30 % by weight of a fatty acid, 1 to 30 % by weight of a fat or an oil and 1 to 30 % by weight of a wax, based on the total weight of the pharmaceutical preparation; or

the carrier of the physiologically active substance comprises about 50 % by weight of hyaluronic acid and about 50 % by weight of a cationic polyacrylic acid derivative, based on the total weight of the pharmaceutical preparation.

As still another embodiment, this invention is directed to a method for prophylaxis or treatment of cerebral vasospasm which comprises administering an effective amount of at least one of MAXs into the body.

As still another embodiment, this invention is directed to a method for prophylaxis or treatment of cerebral vasospasm by use of a compound having a vasodilative action as a physiologically active substance, which comprises a step to implant a prolonged releasing (gradually releasing) pharmaceutical preparation carrying the compound into the brain.

As still another embodiment, this invention is directed to the use of a compound having a vasodilative action for preparing an intrathecal implantation-type prolonged releasing pharmaceutical preparation for prophylaxis or treatment of cerebral vasospasm.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a graph showing the behaviors of release of CGRP from pharmaceutical preparation of the invention, P-I, P-II and P-III (prepared in Example 1, Example 2 and Example 3, respectively) in the in vitro test;

Fig. 2 is a graph showing the behaviors of release of maxadilan (SEQ ID NO : 3) from pharmaceutical preparations of the invention, P-VII, P-VIII and P-IX (prepared in Example 7, Example 8 and Example 9, respectively) in the in vitro test;

Fig. 3 is a graph showing the behavior of release of CGRP from a pharmaceutical preparation of the invention, P-XI (prepared in Example 11);

Fig. 4 is a graph showing the behaviors of release of CGRP from pharmaceutical preparations of the invention, P-XIII and P-XIV (prepared in Example 13 and Example 14, respectively) in the in vitro test;

Figs. 5, and 6 to 8 are a graph showing the behaviors of release of CGRP from pharmaceutical preparations of the invention, P-XV-1 and P-XV-2 (both prepared in Example 15), and P-XVI to P-XX (prepared in Examples 16 to 20), in the in vitro test;

Fig. 9 is a graph showing the behavior of release of CGRP from a pharmaceutical preparation of the invention, P-II, implanted into the brain of a rabbit;

Figs. 10 and 11 show results obtained by implanting preparations of the invention, P-V and P-VI, into the brain, particularly at the subarachnoid region of model animals of subarachnoid hemorrhage, respectively, and observing the behaviors of vasodilative;

Fig. 12 is a graph showing change of the CGRP concentration in the CSF when an aqueous CGRP solution was administered to a rabbit by a cisternal puncture method, and showing stability of CGRP in the CSF;

Fig. 13 shows the results of the vasodilative test by administration of the aqueous CGRP solution by the cisternal puncture method;

Fig. 14 shows the results of the vasodilative test similar to those in Figs. 10 and 11, on a preparation of the invention, P-VIII (containing an N-terminus-modified maxadilan, SEQ ID NO: 3);

Fig. 15 is a graph showing the results of an in vitro release test on a preparation of the invention, P-II by change of the CGRP concentration in the CSF; and

Fig. 16 is a graph showing the effect of inhibition of vasospasm when an aqueous solution of a modified-type maxadilan, SEQ ID NO: 3 was administered to a model animal of cerebral vasospasm by a cisternal puncture method.

DETAILED DESCRIPTION OF THE INVENTION

Cellulosic polymers used in the invention include, for example, cellulose ether derivatives such as hydroxypropylcellulose, methylcellulose, hydroxypropylmethylcellulose, hydroxyethylcellulose and carboxymethylcellulose; and crystalline cellulose. Preferred among the cellulose ether derivatives is hydroxypropylcellulose. The compounding amount of this polymer in the pharmaceutical preparation cannot be limited since it varies depending on the kinds of auxiliaries to be combined and physiologically active substances to be carried thereon, and is determined taking release initiation time and release sustainment time into account, but is generally 10 to 90 % by weight, preferably 40 to 60 % by weight, based on the total weight of the pharmaceutical preparation.

Fat and oils or waxes include hardened oils, cacao butter, beef tallow, lard, beeswax, carnauba wax, white wax, etc. Preferred among them are hardened oils. The compounding amount thereof is not limitative, either, and can be determined taking release initiation time and release sustainment time into account, but is generally 1 to 30 % by weight, preferably 10 to 20 % by weight, based on the total weight of the pharmaceutical preparation. Hereafter, expressions of % by weight are based on the total weights of the pharmaceutical preparations, respectively, unless otherwise defined.

Fatty acids include saturated or unsaturated carboxylic acids having 12 to 22 carbon atoms such as stearic acid, lauric acid, myristic acid, isostearic acid, palmitic acid and behenic acid. Stearic acid is preferred among them. The compounding amount thereof is not limitative, and can also be determined taking release initiation time and release sustainment time into account, but is generally 1 to 30 % by weight, preferably 10 to 20 % by weight.

Saccharides include sucrose, lactose, glucose, fructose, maltose, dextrin, trehalose, pullulan, etc. Preferred among them are lactose and glucose. The disintegrability of the gradually releasing preparation can be adjusted by addition of a saccharide. Namely, the disintegration velocity can be accelerated by increasing its compounding amount. The compounding amount is not limitative, and can also be determined taking release initiation time and release sustainment time into account, but is generally 1 to 40 % by weight, preferably 10 to 30 % by weight. When a saccharide is used as an auxiliary, especially in the case where it is combined with the above cellulose ether derivative, desired effects can be obtained without incorporating a fat or oil or a wax, or a fatty acid, which is an auxiliary component.

When crystalline cellulose is used as a cellulosic polymer, a combination thereof with a polyacrylate ester derivative such as poly (methacrylic acid-co-ethyl acrylate), poly (methacrylic acid-co-methyl methacrylate) and poly (methyl methacrylate-co-ethyl acrylate), preferably Eudragid L30D-5.5, and L100 (trade name; available from Lame Co., Germany) is recommended. The compounding amount of the polyacrylate ester derivative is generally 0.01 to 10 % by weight, preferably 0.1 to 5 % by weight.

As a carrier suitably usable in combination with CGRP or MAXs among later-described physiologically active substances, an ion complex comprising hyaluronic acid and a cationic polyacrylic acid derivative can also be mentioned. As to hyaluronic acid, any of those (straight-chain high molecular polysaccharides formed through alternate bonds derived from β-N-acetyl-D-glucosamine and β-D-glucuronic acid) derived from wide natural origins, for example, the connective tissues of mammals, cockscombs of chickens, capsules of streptococci, etc. can also be used. Suitable cationic polyacrylic acid derivatives such as poly

(methyl methacrylate-co-butyl methacrylate-co-dimethylamino-ethyl methacrylate) and poly (ethyl acrylate-co-methyl methacrylate-co-trimethylaminonium ethyl methacrylate hydrochloride) include, for example, Eudragid E and Eudragid ES (trade names; available from Lame Co., Germany). As to the rate of mixing of them, they are suitably used almost in equal amounts.

Various physiologically active substances can be used, without particular limitation about kinds and action patterns contemplated thereon, so long as they can be prepared as a pharmaceutical preparation of the invention and can attain significant effects through continuous release. Such physiologically active substances include, for example, adrenaline, abscisic acid, arginine vasotocin, angiotensinogen, angiotensin, angiotensin I converting enzyme, succus gastricus-inhibiting polypeptides, insulin, insulin-like growth factors, S factor, erythropoietin, luteinizing hormone, luteinizing hormone-releasing hormone, progestogen, oxytocin, 2-octyl-γ-bromoacetoacetate, autacoids, gastrin, gastrin secretion-accelerating peptide, gastron, activated vitamin D_3, kallidin, calcitonin, calcitonin gene-related peptide (CGRP), kininogen, thymus hormone, glucagon, glucocorticoids, vasoactive small intestinal peptide, plasma kallikrein, serum factor, blood glucose-elevating hormone, thyroid-stimulating hormone, thyrotropin-releasing hormone, thyroid hormone, melanosite-stimulating hormone, melanosite-stimulating hormone-releasing hormone, melanosite-stimulating hormone release-inhibiting hormone, corticotropin-like middle lobe peptide, urokinase, cholecystokinin octapeptide, cholecystokinin tetrapeptide, cholecystokinin variant, cholecystokinin-12, cholecystokinin pancreothymine, cholecystokinin, growth factor, substance P, female sex hormones, adipokinin, chorionic gonadotropin, nerve growth factor, pancreatic polypeptides, reproduction nest-stimulating substance, gonadotropic hormones, growth hormone, growth hormone-releasing factor, secretin, caerulein, serotonin, fibroblast growth factor, kallikrein glandularis, somatostatin, somatomedins A and B, placental lactogen, thymosin, thymopoietin, thyroglobulin, traumatic acid, endothelial cell growth factor, mollusc heart stimulant nervous peptide, neurotensin, equine serum gonadotropic hormone, brain hormones, noradrenaline, vasopressin, estrogenic hormone, histamine, epidermic cell growth factor, parathyroid hormone, parathyroid-stimulating hormone, corticotropin-releasing factor, adrenal cortical hormone, PACAP, bradykinin, bradykinin-like peptide, proinsulin, proopiomelanocortin, prostaglanadins, pro PTH, prolactin, prolactin-releasing hormone, prolactin release-inhibiting hormone, florigene, human menopausal gonadotropin, bombesine, maxadilans (MAXs), mineral corticoid, light-adapted horomone, methionyllysylbradykinin, 1-methyladrenine, melatonin, motilin, androgen, diuretic hormone, lipotropin, renin, relaxin and follicle maturation hormone.

Among physiologically active substances including those enumerated above, those usable for prophylaxis or treatment of cerebral vasospasm, which is a preferred embodiment of the invention, specifically include CGRP, MAXs, deferoxamine, methylprednisolone, nicorandil, nicaraben, magnesium sulfate, actinomycin D, 21-amino-steroid, isoproterenol, tPA, nimodipine, hydrocortisone, nicardipine, nifedipine, diltiazem, dilazp, teprothid, AA861, papaverine, OKY 1581, amyl nitrite, erythrityl tetranitrate, isosorbide dinitrate, nitroglycerin, pentaerythritol tetronitrate, VIP, vasopressin, bradykinin, PACAP, SOD, catalase, bepridil, nadololol, felodipine, isradipine, varapamil, atenolol, metoprolol and propanolol.

Among them, compounds having a vasodilative action, particularly CGRP and MAXs can be mentioned as those which exert significant effects, particularly in prophylaxis or treatment of cerebral vasospasm, in combination with carriers in accordance with the invention of the present application, or in combination with other carriers capable of continuously releasing physiologically active substances, specifically by intracorporeal implantation, particularly by intrathecal implantation.

The abbreviation of MAXs is used herein in a conception of including a natural-occurring peptide (or protein) (SEQ ID NO: 1) derived from the sand fly Lutzomyia longipalpis disclosed in above-mentioned E. A. Lerner et al., WO 91/00293 and its recombinant peptides; and the GIL-modified maxadilan (see, SEQ ID NO: 2; E. A. Lerner et al., J. Bio. Chem., Vol. 267 (2), pp. 1062-1066, 1992) wherein its N-terminus is modified with a sequence consisting of three amino acid residues, GIL-; and their analogues. A prepresentative analogue includes a peptide fragment obtained, according to the method disclosed in E. A. Lerner et al., ibid., namely by obtaining a particular modified-type maxadilan-fused protein, and then disgesting it with a protease such as factor Xa or thrombin. Representative among them are a peptide (SEQ ID NO: 3) wherein an amino acid sequence residue GSIL- is bonded to the N-terminus of SEQ ID NO: 1 maxadilan, and a peptide (SEQ ID NO: 4) wherein an amino acid sequence residue LVPRGSIL- is bonded thereto. MAXs also include those wherein one or more amino acid residues in the amino acid sequence are deleted or replaced, and those wherein one or more amino acid residues are added to the N-terminus or C-terminus, and may be converted amino acid residues - Lys Ala Gly Lys at the C-terminus thereof to - Lys Ala-NH_2.

A person skilled in the art will be able to obtain MAXs usable in the invention, referring to the amino acid sequences specifically disclosed in SEQ ID NOs.: 1 to 4 of the above Sequence Listing, by a liquid

phase or solid phase peptide synthesis method known per se, or by a recombinant method wherein a nucleotide sequence is used which encodes a sequence formed by deleting one or more amino acid residues in its amino acid sequence or their amino acid sequences, or replacing them by other amino acid residue(s), or adding other amino acid residue(s) into the sequence(s).

The level of a physiologically active substance which can be contained in the pharmaceutical preparations of the invention composed of the above-mentioned carriers and optionally used auxiliaries is not limited since the optimal amount varies depending on kinds of carriers and active substances to be used, and methods for application of the preparations. However, generally, it is possible to incorporate 1 x 10^{-12} to 30 % by weight, preferably 1 x 10^{-4} to 5 % by weight of a physiologically active substance, based on the total weight of the pharmaceutical preparation, into the preparation.

The pharmaceutical preparations of the invention can be prepared by compounding the above-mentioned carrier-constituting components and physiologically active substances at levels described above, respectively, and using formulation techniques known per se. In such formulation, it is possible to incorporate one or more of optional additives conventionally used in the art, for example, disintegration-adjusting agents, stabilizers, antioxidants, wetting agents, binders, lubricants, etc., in accordance with the forms of use of the pharmaceutical preparations. The dosage forms of preparations thus prepared can usually be tablets, pills or capsules. However, the dosage form of the preparations may also be a liquid obtained by pulverizing a solid agent prepared above so that it can be used as an injection, and suspending the powder in a suitable fluid (e.g., sterilized distilled water, physiological saline, etc.).

On the other hand, it is known that part of MAXs among the above physiologically active substances have a vasodilative action, as is the case with CGRP, as disclosed by the above E. A. Lerner et al. For example, the maxadilan of SE ID NO: 2 exhibits an extremely interesting vasodilative action 80 to 100 times higher than that of CGRP. An N-terminus-modified-type maxadilan denoted by SEQ ID NO: 3 is known to exhibit a vasodilative action (particularly, erythema activities) further about 10 times higher than that of SEQ ID NO: 2 (the above M. Ohnuma et al., Peptide Chemistry 1993 : Y. Okada (Ed.), pp. 145-148), and is a particularly interesting peptide.

However, it has not so far been disclosed in technical literatures that these MAXs can be used for prophylaxis or treatment of cerebral vasospasm.

Thus, according to this invention, although cerebral vasospasm can be prevented or treated by implanting a pharmaceutical preparation comprising the above carriers having contained therein at least one of MAXs, preferably into a living body, particularly into the brain, a method to use MAXs for treating the disease without using these carriers is also disclosed.

Namely, this invention also provides a method for prophylaxis or treatment of cerebral vasospasm which comprises a step of administering an effective amount of at least one of MAXs into the body of a patient to whom cerebral vasospasm may occur or a patient to whom cerebral vasospasm occurred. This administration step can, for example, be performed by administering an injection obtained by merely dissolving or suspending at least one of MAXs in sterilized distilled water, physiological saline or a buffered solution, into the vein or artery, or can also be performed by administering preparations obtained by adding various inorganic salts as an ionic strength-adjusting agent, and other excipients, for example, dextrin, lactose, starch, etc. thereto, and formulating the mixtures.

Administration time varies depending on dosage forms adopted, administration routes, and the purpose of use (prophylaxis or treatment), but can usually be immediately to 10 days after the operation of subarachnoid hemorrhage.

Thus, this invention also provides as another embodiment the use of at least one of MAXs for preparing a pharmaceutical preparation for prophylaxis or treatment of cerebral vasospasm. The use of prolonged releasing pharmaceutical preparations of CGRP or MAXs for intrathecal implantation has not so far been disclosed in technical literatures, either, and, in addition, has a significantly excellent advantage, for example, over administration of a sterile aqueous solution of CGRP into the cisterna magna, as disclosed in the above H. Shimizu et al., No Shinkei Geka, 22(2) : 131-139, 1994.

A further important thing is that effective prophylaxis or treatment of cerebral vasospasm can be performed not only by combinations of the above-mentioned carriers for pharmaceutical preparations with CGRP or MAXs, but also by implanting into the brains pharmaceutical preparations prepared by combinations of physiologically active substances exhibiting a vasodilative action, as mentioned above with other carriers.

Thus, from this viewpoint, as stated above, this invention also provides a method for prophylaxis or treatment of cerebral vasospasm which comprises a step of implanting a prolonged releasing pharmaceutical preparation comprising certain carriers having contained therein a physiologically active substance having a vasodilative action, preferably at least one selected from the group consisting of CGRP and MAXs,

into the brain of a patient to whom cerebral vasospasm may occur or a patient to whom cerebral vasospasm occurred. As an alternative embodiment of this method, this invention also provides the use of a physiologically active substance (or compound) having a vasodilative action, preferably at least one selected from the group consisting of CGRP and MAXs, for preparing an intrathecal implantation-type pharmaceutical preparation for prophylaxis or treatment of cerebral vasospasm.

The pharmaceutical preparation of the invention of the present application can be administered in a predetermined effective amount in a prolonged releasable form, particularly parenterally, but preferably, the characteristic of the preparation lies in a point that it exhibits a remarkable effect when implanted into a living body, particularly into the brain. The pharmaceutical preparation of the invention, when thus implanted into the brain, prolonged releases the active substance with retention of the dosage form for 7 days or more, mostly 10 days or more, and thus the active substance distributes only on the administration site and does not disperse throughout the brain. Therefore, there is not waste of the active substance, and moreover, there is less possibility that it has unnecessary actions on other sites. Moreover, the preparation has desired properties that it does not hurt the cells at the administration site, and releases that active substance stably over a long term as long as 2 days to 2 weeks after the administration, and thereafter the base (carriers and auxiliaries) is absorbed in the living body. When the preparation is intrathecally implanted as a tablet, it is desirable to place it in the subarachnoid space and/or in the furrow of the brain surface so that it is not let to flow by the reflux of the cerebrospinal fluids. It is also desirable that the thickness of the preparation is 5 mm or less so that it can be intrathecally implanted without any trouble, and since when the expansion coefficient of the tablet is too large, it may do damages such as cell detachment to the cells and/or tissues around the administration site, and therefore, it is desirable to adjust the carrier components and the auxiliary components to make the expansion coefficient 200 % or less.

A person skilled in the art will be able to readily determine the optimal amounts of physiologically active substances which can be incorporated in these prolonged releasing pharmaceutical preparations, according to in vitro tests or in vivo tests as described later.

This invention is further illustrated below according to specific examples, but it should not be construed that they are provided for the purpose of limiting the scope of the invention.

Example 1

Preparation of a prolonged releasing pharmaceutical preparation (P-I)

10 g of stearic acid and 19 g of hardened oil were mixed, 2.0 g of 0.4 % CGRP solution (corresponding to 8 mg of CGRP) and 20 g of lactose were added thereto and the mixture was mixed, and 60 g of hydroxypropylcellulose was added thereto. The mixture was mixed adequately, and then subjected to a KBr compressor (150 kg, 1 minute) to prepare tabular tablets (P-I) having a diameter of 13 mm.

Example 2

Preparation of a prolonged releasing pharmaceutical preparation (P-II)

20 g of stearic acid and 20 g of hardened oil were mixed, 2.5 g of 0.4 % CGRP solution (corresponding to 10 mg of CGRP) and 20 g of lactose were added thereto and the mixture was mixed, and 40 g of hydroxypropylcellulose was added thereto. The mixture was mixed adequately, and then pressure molded using a Correct 19 K compressor (KIKUSUI CLEAN PRESS) (6 mmØ x 2 mm).

Example 3

Preparation of a prolonged releasing pharmaceutical preparation (P-III)

15 g of palmitic acid and 15 g of beeswax were mixed, 2.0 g of 0.4 % CGRP solution (corresponding to 8 mg of CGRP) was added thereto and the mixture was mixed, and 70 g of hydroxypropylcellulose was added thereto. The mixture was mixed adequately, and then pressure molded using a Correct 19 K compressor (KIKUSUI CLEAN PRESS) (6 mmØ x 2 mm).

Example 4

Preparation of a prolonged releasing pharmaceutical preparation (P-IV)

10 g of stearic acid and 10 g of hardened oil were mixed, 2.0 g of 0.4 % CGRP solution (corresponding to 8 mg of CGRP) and 20 g of glucose were added thereto and the mixture was mixed, and 60 g of hydroxypropylcellulose was added thereto. The mixture was mixed adequately, and then pressure molded using a Correct 19 K compressor (KIKUSUI CLEAN PRESS) (6 mmØ x 2 mm).

Example 5

Preparation of a prolonged releasing pharmaceutical preparation (P-V)

10 g of stearic acid and 10 g of hardened oil were mixed, 2.5 g of 1.6 % CGRP solution (corresponding to 40 mg of CGRP) and 20 g of lactose were added thereto and the mixture was mixed, and 60 g of hydroxypropylcellulose was added thereto. The mixture was mixed adequately, and then pressure molded using a Correct 19 K compressor (KIKUSUI CLEAN PRESS) (6 mmØ x 2 mm).

Example 6

Preparation of a prolonged releasing pharmaceutical preparation (P-VI)

10 g of stearic acid and 10 g of hardened oil were mixed, 2.5 g of 10 % CGRP solution (corresponding to 250 mg of CGRP) and 20 g of lactose were added thereto and the mixture was mixed, and 60 g of hydroxypropylcellulose was added thereto. The mixture was mixed adequately, and then pressure molded using a Correct 19 K compressor (KIKUSUI CLEAN PRESS) (6 mmØ x 2 mm).

Example 7

Preparation of a prolonged releasing pharmaceutical preparation (P-VII)

10 g of stearic acid and 10 g of hardened oil were mixed, 2.5 g of 0.6 % solution of a GSIL-modified-type maxadilan denoted by SEQ ID NO: 3 (corresponding to 15 mg of SEQ ID NO: 3) and 20 g of lactose were added thereto and the mixture was mixed, and 60 g of hydroxypropylcellulose was added thereto. The mixture was mixed adequately, and then pressure molded using a Correct 19 K compressor (KIKUSUI CLEAN PRESS), and the moldings were pulverized and mixed and pressure molded again using the same compressor (6 mmØ x 2 mm).

Example 8

Preparation of a prolonged releasing pharmaceutical preparation (P-VIII)

10 g of stearic acid and 10 g of hardened oil were mixed, 2.5 g of 1.0 % solution of a GSIL-modified-type maxadilan denoted by SEQ ID NO: 3 (corresponding to 25 mg of SEQ ID NO: 3) and 20 g of lactose were added thereto and the mixture was mixed, and 60 g of hydroxypropylcellulose was added thereto. The mixture was mixed adequately, and then pressure molded using a Correct 19 K compressor (KIKUSUI CLEAN PRESS), and the moldings were pulverized and mixed and pressure molded again using the same compressor (6 mmØ x 2 mm).

Example 9

Preparation of a prolonged releasing pharmaceutical preparation (P-IX)

10 g of stearic acid and 10 g of hardened oil were mixed, 2.5 g of 10 % solution of a GSIL-modified-type maxadilan denoted by SEQ ID NO: 3 (corresponding to 250 mg of SEQ ID NO: 3) and 20 g of lactose were added thereto and the mixture was mixed, and 60 g of hydroxypropylcellulose was added thereto. The mixture was mixed adequately, and then pressure molded using a Correct 19 K compressor (KIKUSUI CLEAN PRESS), and the moldings were pulverized and mixed and pressure molded again using the same

compressor (6 mmØ x 2 mm).

Example 10

Preparation of a prolonged releasing pharmaceutical preparation (P-X)

15 g of palmitic acid and 15 g of beeswax were mixed, 3.0 g of 0.4 % solution of a GSIL-modified-type maxadilan denoted by SEQ ID NO: 3 (corresponding to 10 mg of SEQ ID NO: 3) was added thereto and the mixture was mixed, and 70 g of hydroxypropylcellulose was added thereto. The mixture was mixed adequately, and then pressure molded using a Correct 19 K compressor (KIKUSUI CLEAN PRESS) (6 mmØ x 2 mm).

Example 11

Preparation of a prolonged releasing pharmaceutical preparation (P-XI)

2.0 g of 0.4 % CGRP solution (corresponding to 8 mg of CGRP) and 20 g of lactose were mixed adequately, and 80 g of hydroxypropylcellulose was added thereto. The mixture was mixed adequately, and then subjected to a KBr compressor (150 kg, 1 minute) to prepare tabular tablets (P-XI) having a diameter of 13 mm.

Example 12

Preparation of a prolonged releasing pharmaceutical preparation (P-XII)

2.5 g of 0.4 % CGRP solution (corresponding to 10 mg of CGRP) and 20 g of glucose were mixed adequately, and 80 g of methylcellulose was added thereto. The mixture was mixed adequately, and then subjected to a KBr compressor (150 kg, 1 minute) to prepare tabular tablets (P-XII) having a diameter of 13 mm.

Example 13

Preparation of a prolonged releasing pharmaceutical preparation (P-XIII)

2.5 g of 0.4 % CGRP solution (corresponding to 10 mg of CGRP) and 13 g of poly (methacrylic acid-co-methyl methacrylate), Eudragid L-100 (trade name; available from Lame Co., Germany), were mixed, and 87 g of crystalline cellulose was added thereto. The mixture was mixed adequately, and then subjected to a KBr compressor (150 kg, 1 minute) to prepare tablets (P-XIII) having a diameter of 13 mm.

Example 14

Preparation of a prolonged releasing pharmaceutical preparation (P-XIV)

17 g of stearic acid and 17 g of hydrogenated oil (hydrogenated castor oil) were mixed, 2.0 g of 0.4 % CGRP solution (corresponding to 8 mg of CGRP) and 0.3 g of poly (methacrylic acid-co-ethyl acrylate), of Eudragid L30D-5.5 (trade name; available from Lame Co., Germany), were added and the mixture was mixed, and 66 g of crystalline cellulose was added thereto. The mixture was mixed adequately, and then subjected to a KBr compressor (150 fkg/cm^2, 1 minute) to prepare tablets (P-XIV) having a diameter of 13 mm.

Example 15

Preparation of a prolonged releasing pharmaceutical preparation (P-XV)

100 g of aqueous 2 % hyaluronic acid solution and 100 g of aqueous 2 % Eudragid E solution were subjected to reaction at room temperature for 2 hours under stirring. The reaction mixture was centrifuged at 3,000 rpm for 10 minutes, and the product was recovered and vacuum dried to give a polyion complex of hyaluronic acid Eudragid E.

The obtained solid was pulverized and classified to give powder having a particle size of 150 microns or less. 100 g of the powder and 0.75 of 0.4 % CGRP solution (corresponding to 3 mg of CGRP) were mixed, and then pressure molded using a Correct 19 K compressor (KIKUSUI CLEAN PRESS), and the moldings were pulverized and mixed and pressure molded again using the same compressor (6 mmØ x 2 mm).

Examples 16 to 20

Procedures described in the aforementioned Examples, particularly Examples 14 and 15, are repeated, except that compositions of the preparation are those used in the following table 1.

Table 1

Compositions	Example Nos. (preparation Nos.) 16 (P-XVI)	17 (P-XVII)	18 (P-XVIII)	19 (P-XIX)	20 (P-XX)
Crystalline cellulose (mg)	1600	1450	1300	2000	0
Hydrogenated oil (hydrogenated caster oil) (mg)	200	270	330	0	0
Stearic acid (mg)	200	270	330	0	0
Eudragit® L30D-5.5 (µl)	120	110	100	300	300
Eudragit® L100 (g)	0	0	0	0.4	0.2
CMC·Na (g)	0	0	0	0	1.5
Lactose (g)	0	0	0	0	0.5
CGRP (mg)	0.2	0.2	0.2	0.2	0.2
H_2O (ml)	0.2	0.2	0.2	0.2	0.2
Ethanol (ml)	0	0	0	5	0

Examples 21 to 34

In vitro release test on physiologically active substances

5 ml portions of Hartmann's Solution (available from The Green Cross Corp., Japan) were aseptically put in 15-ml tubes, respectively.

The following pharmaceutical preparations were aseptically put in these tubes, respectively:

P-I, P-II, P-IV (Fig. 1)
P-VII, P-VIII, P-IX (Fig. 2)
P-XI (Fig. 3)
P-XIII, P-XIV (Fig. 4)
P-XV (Fig. 5)
P-XVI, P-XVII, P-XIX (Fig. 6)
P-XVIII (Fig. 7)
P-XX (Fig. 8)

The mixtures were shaken at 37 °C and 120 rpm, sampling was performed 1, 2, 3, 7, 11 and 14 days thereafter, and each sample was subjected to determination of the release amount of the physiologically active substance by high performance liquid chromatography. The results are shown in Figs. 1 to 8, respectively (The relations between the figures and the pharmaceutical preparations are shown in the above parentheses).

Numbers in the figures express the disintegration states of the tablets, and the meanings are as follows:

Disintegration state

1 : No change
2 : 20 % swelling is observed
3 : Small cracks are formed
4 : Large cracks are formed
5 : Disintegrated into pieces

Example 35

In vivo release test on a physiologically active substance

An in vivo test on drug release effect with 3 rabbits according to the method shown below was performed, using the tablets (P-II) obtained in Example 2. The results are shown in Fig. 9.

Test method

Method of intrathecally implantation of the tablets into the brains

The rabbits (2.5 - 3.0 kg) after sodium pentobarbital anesthesia were fixed on their faces, the occipital bone membranes (dura maters) were exposed by incision, and the occipital bones were shaved by a drill to expose the dura maters further wider. Thereafter, the dura maters and the arachnoidmembrane were incised to a length of about 8 mm, the tablets of the prolonged releasing pharmaceutical preparation of Example 2 were implanted there on two of the three animals, a placebo tablet was implanted there on the other one, the dura maters, the muscles and the skins were sutured, and an appropriate amount of an antibiotic was administered to the incision sites, respectively.

The cerebrospinal fluids were sampled every day from the rabbits by the following sampling method, respectively, and the samples were assayed for the CGRP concentrations (nM) in the cerebrospinal fluids according to the following assay method.

Method of sampling of the cerebrospinal fluids

The rabbits after sodium pentobarbital anesthesia were fixed on their faces, the occipital bone membranes (dura maters) were exposed by incision, the occipital bone membranes were incised, and the celebrospinal fluids were sampled therefrom.

Method of assay of the concentration of the physiologically active substance in the celebrospinal fluids

The concentrations were assayed by the following radioimmunoassay.

4,000 cpm portions of a labeling compound [2-(^{125}I-idohistidyl^{10})CGRP] were put in measuring tubes, and separately from these, 100 µl portions of 1, 2, 5, 10, 50, 100, 500 and 100 fmol standard solutions were prepared using synthesized CGRP (available from Bachem Co.). 100 µl portions of an antibody (obtained by dissolving RPN 1841 available from Amarsham Co. in 2 ml and diluting the solution to 12.5 ml) and 600 µl portions of an analyzing buffer [50 mM sodium phosphate (pH 7.4), 0.3 % bovine serum albumin, 10 mM EDTA] were added, respectively, to the tubes containing 100 µl portions of the test samples, the standard solutions or water, respectively, the covers of the tubes were shut, and the mixtures were allowed to stand at 4 °C for 5 days, 250 µl of a dextran/active carbon solution [50 mM sodium phosphate (pH 7.4), 0.25 % gelatin, 10 mM EDTA] was added to each mixture, and the resultant mixture was immediately centrifuged at 2,000 x g for 20 minutes. Both of the precipitate and the supernatant were measured for 200 seconds by a γ-counter, and the concentration of the physiologically active substance (CGRP) in the cerebrospinal fluid was assayed based on the standard curve obtained from the standard substance solutions.

Separately, an in vivo test related to the disintegration state of a tablet was performed using the tablet of P-II as the tablet and a rabbit other than the above rabbits. In this connection, implantation of the tablet into the brain was performed in the same manner as above, and the disintegration state of the tablet was observed through craniotomy. The results are shown below. The following numerical values exhibit the disintegration state of the tablet, and the meanings are the same as defined above.

After implantation	1st day	5th day	10th day
Disintegration state	2	2	4

It is understood from the results that the prolonged releasing cerebral vasospasm inhibitor of the invention keeps the dosage from in vivo even 10 days after the intrathecal implantation.

Examples 36 and 37

Vasodilative test (I)

A vasodilative test was performed according the following method, using the tablets obtained in Example 5 and Example 6, i.e, P-V and P-VI, respectively, and tablets not containing a physiologically active peptide CGRP (placebo tablets). The results are shown in Fig. 10 about the tablet of P-V and in Fig. 11 about the tablet of P-VI.

Process of preparation of the placebo tablet

Prolonged releasing pharmaceutical preparations as placebos were prepared in the same manners as in the preparation of the prolonged releasing pharmaceutical preparation of Example 5 (P-V) and in the preparation of the prolonged releasing pharmaceutical preparation of Example 6 (P-VI), respectively, except for replacement of CGRP by hydroxypropylcellulose.

Vasodilative test

An experiment was performed according to the following procedure using 8 rabbits about the tablet of P-V and 7 rabbits about the tablet of P-VI, these rabbits weighing 2.5 to 3 kg.

(1) After an X-ray photograph of the basilar artery of each rabbit was taken, subarachnoid hemorrhage models were prepared according to a known method [D. G. Vollmer et al., Neurosurgery 28 : 27-32 (1991)]. (Day 0)

(2) 24 hours later (Day 1), each animal was anesthetized with sodium pentobarbital, and the occipital region was incised from the occipital bone to the primary cervical vertebrae along the median line.

(3) The muscles attached to the occipital bone, the primary cervical vertebrae and the occipital bone membrane were carefully detached by a knife so as not to injure the vein.

(4) The exposed occipital bone was shaved by a surgical drill to a thickness of the order of 2 to 5 mm from the lower part.

(5) Thereafter, the occipital bone membrane was incised by a knife to a length of the order of 8 to 10 mm.

(6) The tablet was placed in the subarachnoid region through this site using a pair of tweezers.
(7) After the insertion of the tablet, the occipital bone membrane was sutured with silk thread.
(8) The cut portion was further shut with Aron Alpha (trade mark) along the suture line.
(9) Thereafter, the muscle and the skin were sutured with silk thread, and an appropriate amount of antibiotic was administered.
(10) The blood vessel calibers were measured every day from immediately after the administration to 5 days thereafter (Day 6) by angiography.

Comparative example

Comparative tests against Example 35 and Examples 36 and 37

As a comparative example, an aqueous CGRP solution was administered to one rabbit by a cisternal puncture method in accordance with the known method in the above (1), and the CGRP concentration (nM) in the cerebrospinal fluid was assayed over the lapse of time in the same manner as in Example 35. The results are shown in Fig. 12.

As another comparative example, portions of an aqueous CGRP solution or portions of distilled water were administered, respectively to rabbit models (2 animals about the aqueous CGRP solution and 4 animals about the distilled water) in which spasm of the basilar artery had been clearly observed after subarachnoid hemorrhage, also by a cisternal puncture method in accordance with the known method in the above (1), and the blood vessel calibers were measured in the same manner as in the Examples 36 and 37. The results are shown in Fig. 13.

Example 38

Vasodilative test (II)

A vasodilative test was performed in the same manner as in Examples 36 and 37 using the tablet obtained in Example 8 (P-VIII) and a tablet (placebo tablet) not containing a GSIL-modified-type maxadilan denoted by SEQ ID NO: 3 as a physiologically active peptide. The results are shown in Fig. 14. The placebo tablet was prepared in the same manner as in Example 8 except for replacement of the GSIL-modified-type maxadilan by hydroxypropylcellulose.

Example 39

In vivo release test on a physiologically active substance

In addition to Example 35, this experiment was performed aiming to obtain statistically more meaningful data. The operations described in Example 35 were repeated except that 30 rabbits (5 animals each for Normal, day 1, day 2, day 3, day 4 and day 5) were used, and the cerebrospinal fluids (CSF) were collected 1 day (day 1), 2 days (day 2), 3 days (day 3), 4 days (day 4) and 5 days (day 5) after the implantation of the tablet (P-II) from 5 animals each for the respective days, and thereby the CGRP concentrations in the CSFs were assayed. The results are shown in Fig. 15.

In the above Normal means a case where the tablet (P-II) was not implanted.

Example 40

In vivo disappearance test on a pharmaceutical preparation

An in vivo test related to the disappearance effect of a prolonged releasing pharmaceutical preparation (tablet) in the brains was performed by the following method, using the tablet (P-II) obtained in Example 2. In this test, 18 rabbits (2 animals each for day 1, day 2, day 3, day 4, day 5, day 10, month 1, month 3 and month 6) were used. The operations and the results are shown below.

Method of implantation of the tablet into the brain

Each of 18 rabbits (2.5 - 3.0 kg) was anesthetized with sodium pentobarbital and fixed on its face, the occipital bone membrane (dura mater) was exposed by incision, and the occipital bone was shaved by a

drill to expose the dura mater further wider. Thereafter, the dura mater and the arachnoidea were incised to a length of about 8 mm, the tablet of Example 2 as a prolonged releasing pharmaceutical preparation was implanted, the dura mater, the muscle and the skin were sutured, and an appropriate amount of an antibiotic was administered to the incision site.

Assay method and assessment criterion

On 1st day, 2nd day, 3rd day, 4th day, 5th day, 10th day, 1st month, 3rd month, 6th month after the implantation of the tablet into the rabbits, the two rabbits each for the respective days were subjected to a craniotomy operation to expose the portion where the tablet was implanted, and observation was performed visually. The assessment criterion was as follows.

- \- The tablet scarcely disappears
- \- Nearly half of the tablet disappeared
- \+ + Almost all the tablet disappeared
- \+ + + The tablet completely disappeared

Test results

Table 2

Days from the day when the tablets was implanted	Assessment of disappearance of the tablet
1st day	-
2nd day	-
3rd day	- ~ +
4th day	+
5th day	+
10th day	+
1st month	+ +
3rd month	+ + +
6th month	+ + +

There was not any rabbit at all which died during the term when the disappearance effect test on this prolonged releasing pharmaceutical preparation (tablet) was performed.

Example 41

Cerebral vasospasm inhibition test on maxadilan (SEQ ID NO: 3)

Preparation of a cerebral vasospasm model animal

A Japanese white rabbit (male, weighing 2-3 kg) was subjected to general anesthesia by injection of sodium pentobarbital through the auricular vein. After the rabbit was retained so as not to move, the surroundings of the punctured vein were disinfected with sterilized ethanol, a self-retaining needle was punctured into the vein, and immediately thereafter, a silicone-made extension tube equipped, at one end, with a three way cock connected at the two ways with 10-ml syringes each containing physiological saline and connected at the residual way with a 10-ml syringe containing sodium pentobarbital was connected to the self-retaining needle (when the blood vessel is thin, the blood vessel is stimulated by fingers to dilate it). After the connection, the physiological saline was flashed and it was confirmed that the needle was in the vein, and then, the sodium pentobarbital was injected into the rabbit so that the amount became 75 mg per kg of the weight of the rabbit. At this time, since the animal went under anesthesia within one minute, the respiratory tract was previously secured so as to make respiration possible.

Insertion of catheter (Seldinger's method)

The femoral region artery was exposed by incision of the femoral region, and a catheter was inserted into the vertebral artery from the exposed portion under transillumination, using a guide wire. Separately,

0.2 ml of heparin was injected so as to prevent formation of thrombus at the time of insertion of the catheter.

Angiography

A spot for angiography was determined by transillumination, the head was fast fixed, back flow of the blood was confirmed, and 0.8 ml of a contrast medium was injected under a certain pressure (2.3 kg/cm^2). At the time when 0.6 ml thereof was injected, a photograph of the blood vessel was taken.

Cisterna puncture

This procedure was performed in common when blood of a test preparation was injected into the rabbit.

The animal was placed on its face, and the head was leaned downward so as to make an angle of 30° against a horizontal plane. After the occipital bone was confirmed, the primary cervical vertebrae was confirmed, and a 26G butterfly needle was stuck at an angle of about 60° between them. When the needle hit the occipital bone, the needle was made vertical to 90°, and punctured further deeply. At this point of time, it was confirmed that the cerebrospinal fluid flew out by reducing pressure. The fresh blood of the artery was injected through this needle at a rate of 1 ml/min in an amount of 1 ml per kg of the weight of the rabbit. After the injection, back flow of the cerebrospinal fluid was again confirmed, and the rabbit was allowed to stand, leaving it intact, for 15 minutes or more. Photographs of the cerebral blood vessel of the animal were taken 3 days after the blood injection.

Administration of a test preparation

Groups of rabbits, one group consisting of 5 animals, were treated as mentioned above, and among them, rabbits were selected wherein when the basilar artery was divided, starting from the junction from the vertebral artery, into three equal parts, and the diameter at the middle point was measured, spasm occurred at a level of 18 % or more. About the selected rabbits, portions of an aqueous solution of a modified-type maxadilan denoted by SE ID NO: 3 or portions of distilled water as a comparative example were administered 3 days after the injection of the blood, respectively. After the administration, angiography of the basilar artery was performed, on each rabbit, over the lapse of time, and the ratio of the diameter of the blood vessel to that before subarachnoid hemorrhage was investigated. As to the model animals, rabbits weighing 2.5 - 3.0 kg were used as follows: 8 animals in the modified-type maxadilan administration group and 4 animals in the sterile water administration group. The dose of the modified-type maxadilan was 7 μg per kg of the weight of the rabbit.

The results are shown in Fig. 16.

As apparent from Fig. 16, in the aqueous modified-type maxadilan solution administration group, spasm was significantly inhibited during from immediately after the administration to about 4 hours thereafter, compared to the sterile water administration group.

Sequence Listing

SEQ ID NO: 1
SEQUENCE LENGTH : 63
SEQUENCE TYPE : amino acid
TOPOLOGY : linear
MOLECULE TYPE : peptide
SEQUENCE

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Cys Asp Ala Thr Cys Gln Phe Arg Lys Ala Ile Asp Asp Cys Gln Lys  16
 1               5                  10                  15
Gln Ala His His Ser Asn Val Leu Gln Thr Ser Val Gln Thr Thr Ala  32
            20                  25                  30
Thr Phe Thr Ser Met Asp Thr Ser Gln Leu Pro Gly Asn Ser Val Phe  48
        35                  40                  45
Lys Glu Cys Met Lys Gln Lys Lys Lys Glu Phe Lys Ala Gly Lys      63
    50                  55                  60
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SEQ ID NO: 2
SEQUENCE LENGTH : 66
SEQUENCE TYPE : amino acid
TOPOLOGY : linear
MOLECULE TYPE : peptide
SEQUENCE

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Gly Ile Leu Cys Asp Ala Thr Cys Gln Phe Arg Lys Ala Ile Asp Asp  16
 1               5                  10                  15
Cys Gln Lys Gln Ala His His Ser Asn Val Leu Gln Thr Ser Val Gln  32
            20                  25                  30
Thr Thr Ala Thr Phe Thr Ser Met Asp Thr Ser Gln Leu Pro Gly Asn  48
        35                  40                  45
Ser Val Phe Lys Glu Cys Met Lys Gln Lys Lys Lys Glu Phe Lys Ala  64
    50                  55                  60
Gly Lys                                                          66
 65
```

SEQ ID NO: 3
SEQUENCE LENGTH : 67
SEQUENCE TYPE : amino acid
TOPOLOGY : linear
MOLECULE TYPE : peptide
SEQUENCE

```
Gly Ser Ile Leu Cys Asp Ala Thr Cys Gln Phe Arg Lys Ala Ile Asp  16
 1               5                  10                  15
Asp Cys Gln Lys Gln Ala His His Ser Asn Val Leu Gln Thr Ser Val  32
            20                  25                  30
Gln Thr Thr Ala Thr Phe Thr Ser Met Asp Thr Ser Gln Leu Pro Gly  48
        35                  40                  45
Asn Ser Val Phe Lys Glu Cys Met Lys Gln Lys Lys Lys Glu Phe Lys  64
    50                  55                  60
Ala Gly Lys                                                      67
 65
```

SEQ ID NO: 4
SEQUENCE LENGTH : 71
SEQUENCE TYPE : amino acid
TOPOLOGY : linear
MOLECULE TYPE : peptide
SEQUENCE

```
Leu Val Pro Arg Gly Ser Ile Leu Cys Asp Ala Thr Cys Gln Phe Arg  16
                5                  10                  15
Lys Ala Ile Asp Asp Cys Gln Lys Gln Ala His His Ser Asn Val Leu  32
            20                  25                  30
Gln Thr Ser Val Gln Thr Thr Ala Thr Phe Thr Ser Met Asp Thr Ser  48
        35                  40                  45
Gln Leu Pro Gly Asn Ser Val Phe Lys Glu Cys Met Lys Gln Lys Lys  64
    50                  25                  60
Lys Glu Phe Lys Ala Gly Lys                                      71
 65                  70
```

Claims

1. A pharmaceutical preparation carrying an effective amount of a physiologically active substance and capable of prolonged releasing the physiologically active substance, characterized in that the carrier of the physiologically active substance comprises a combination of a cellulosic polymer and at least one auxiliary component selected from fats and oils, waxes, fatty acids, saccharides and polyacrylate ester

derivatives.

2. A pharmaceutical preparation according to claim 1, characterized by being directed to intracorporeal implantation.

3. A pharmaceutical preparation according to claim 1 or 2, characterized in that said intracorporeal is intrathecal.

4. A pharmaceutical preparation according to any of claims 1 to 3, characterized in that the carrier comprises 10 to 90 % by weight of a cellulose ether derivative, 1 to 30 % by weight of a fat or oil or the wax and 1 to 30 % by weight of a fatty acid, based on the total weight of the pharmaceutical preparation.

5. A pharmaceutical preparation according to any of claims 1 to 3, characterized in that the carrier comprises 10 to 90 % by weight of a cellulose ether derivative and 1 to 40 % by weight of a saccharide, based on the total weight of the pharmaceutical preparation.

6. A pharmaceutical preparation according to any of claims 1 to 3, characterized in that the carrier comprises 10 to 90 % by weight of crystalline cellulose and 0.01 to 10 % by weight of a polyacrylate ester, based on the total weight of the pharmaceutical preparation.

7. A pharmaceutical preparation according to any of claims 1 to 6, characterized in that the physiologically active substance is a physiologically active peptide.

8. A pharmaceutical preparation according to claim 7, characterized in that the physiologically active peptide is a calcitonin gene-related peptide (CGRP).

9. A pharmaceutical preparation according to claim 7, characterized in that the physiologically active peptide is any one of maxadilans (MAXs).

10. A pharmaceutical preparation carrying an effective amount of a physiologically active substance and capable of prolonged releasing the physiologically active substance, wherein the physiologically active substance is one or more selected from the group consisting of CGRP and MAXs, and
the carrier of the physiologically active substance comprises about 50 % by weight of hyaluronic acid and about 50 % by weight of a cationic polyacrylic acid derivative, based on the total amount of the pharmaceutical preparation.

11. The use of at least one of MAXs for preparing a pharmaceutical preparation for prophylaxis or treatment of cerebral vasospasm which comprises an effective amount of a physiologically active substance and carrier(s).

12. The use according to claim 11 wherein at least one of MAXs is a maxadilan denoted by SEQ ID NO: 3.

13. The use of a compound having a vasodilative action for preparing an intrathecal implantation-type prolonged releasing pharmaceutical preparation for prophylaxis or treatment of cerebral vasospasm.

14. The use according to claim 13, characterized in that the compound is selected from the group consisting of CGRP, MAXs, deferoxamine, methylprednisolone, nicorandil, nicaraben, magnesium sulfate, actinomycin D, 21-aminosteroid, isoproterenol, tPA, nimodipine, hydrocortisone, nicardipine, nifedipine, diltiazem, dilazp, teprothid, AA861, papaverine, OKY 1581, amyl nitrite, erythrityl tetranitrate, isosorbide dinitrate, nitroglycerin, pentaerythritol tetronitrate, VIP, vasopressin, bradykinin, PACAP, SOD, catalase, bepridil, nadololol, felodipine, isradipine, varapamil, atenolol, metoprolol and propanolol.

15. The use according to claim 14, characterized in that the compound is selected from the group consisting of CGRP and MAXs.

16. The use according to claim 15, characterized in that the compound is at least one of MAXs.

17. The use according to claim 15, characterized in that the compound is CGRP.

18. The use according to claim 16, characterized in that the compound is a maxadilan denoted by SEQ ID NO: 3.

19. The use according to any of claims 13 to 18, characterized in that the carrier of the pharmaceutical preparation comprises 10 to 90 % by weight of a cellulose ether derivative, 1 to 30 % by weight of a fat or oil or a wax, and 1 to 30 % by weight of a fatty acid, based on the total weight of the pharmaceutical preparation.

20. The use according to any of claims 13 to 18, characterized in that the carrier of the pharmaceutical preparation comprises 10 to 90 % by weight of a cellulose ether derivative and 1 to 40 % by weight of a saccharide, based on the total weight of the pharmaceutical preparation.

21. The use according to any of claims 13 to 18, characterized in that the carrier of the pharmaceutical preparation comprises 10 to 90 % by weight of crystalline cellulose and 0.01 to 10 % by weight of a polyacrylate ester, based on the total weight of the pharmaceutical preparation.

22. The use according to any of claims 13 to 18, characterized in that the carrier of the pharmaceutical preparation comprises about 50 % by weight of hyaluronic acid and about 50 % by weight of a cationic polyacrylic acid derivative, based on the total weight of the pharmaceutical preparation.

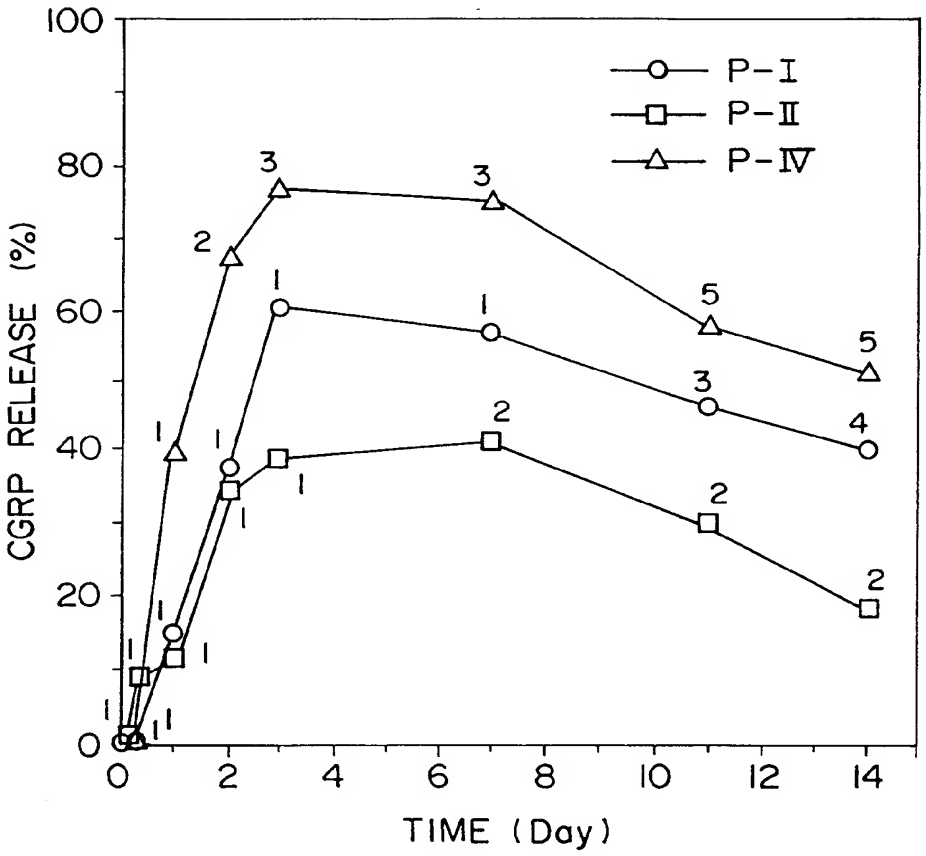

FIG. 1

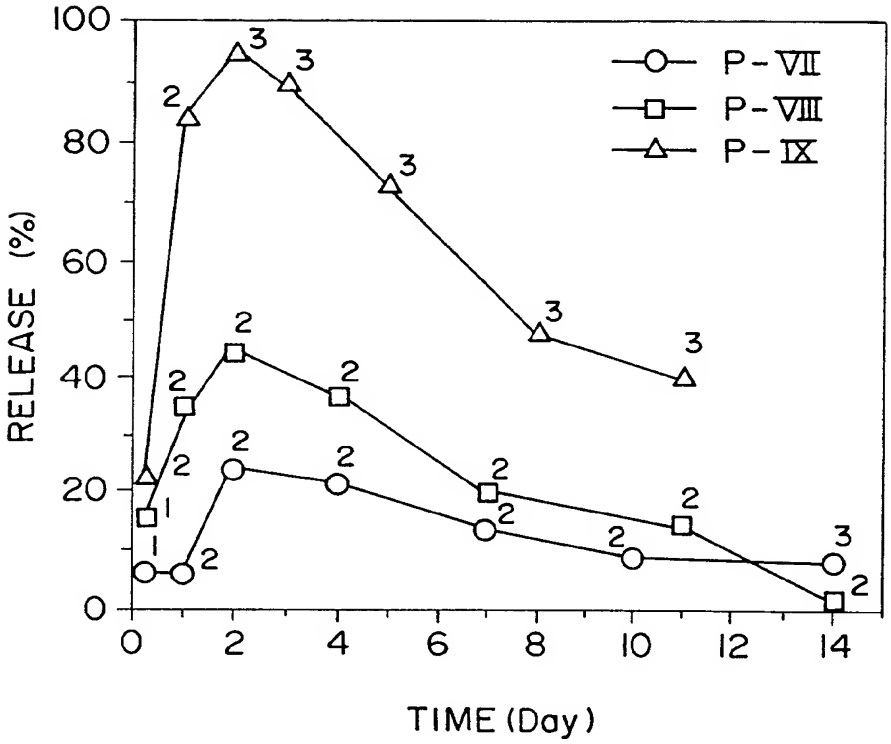

FIG. 2

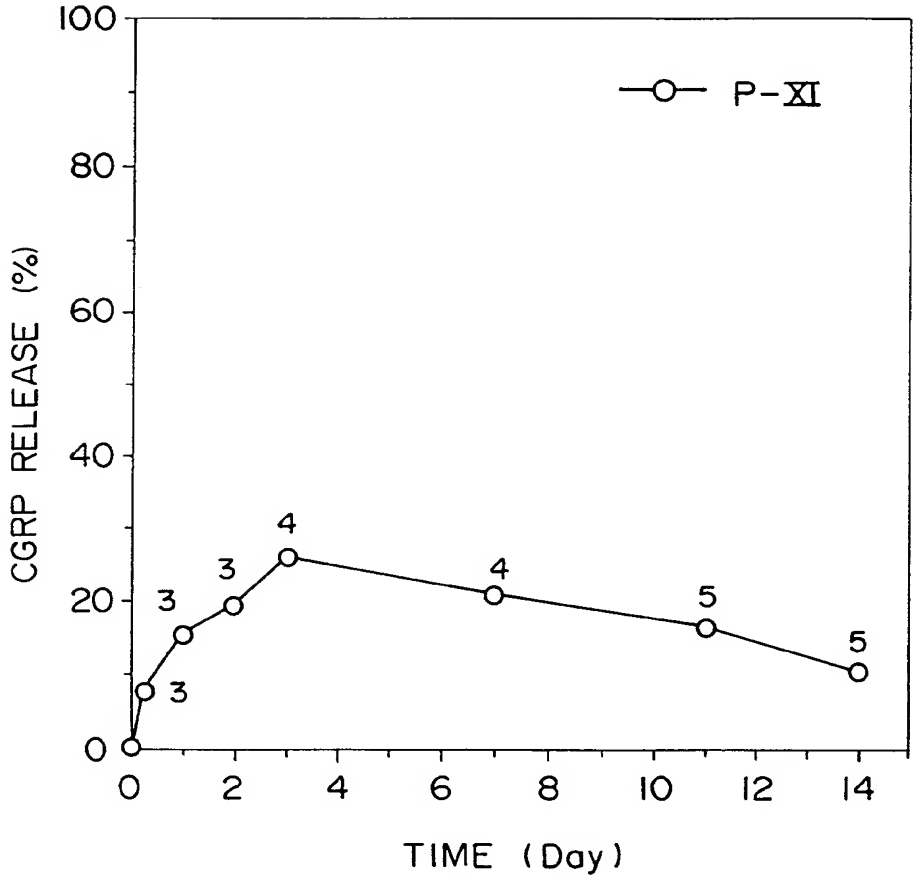
100
80
60
40
20
0
CGRP RELEASE (%)
P-XI
3
3
4
3
4
5
5
0
2
4
6
8
10
12
14
TIME (Day)

FIG. 3

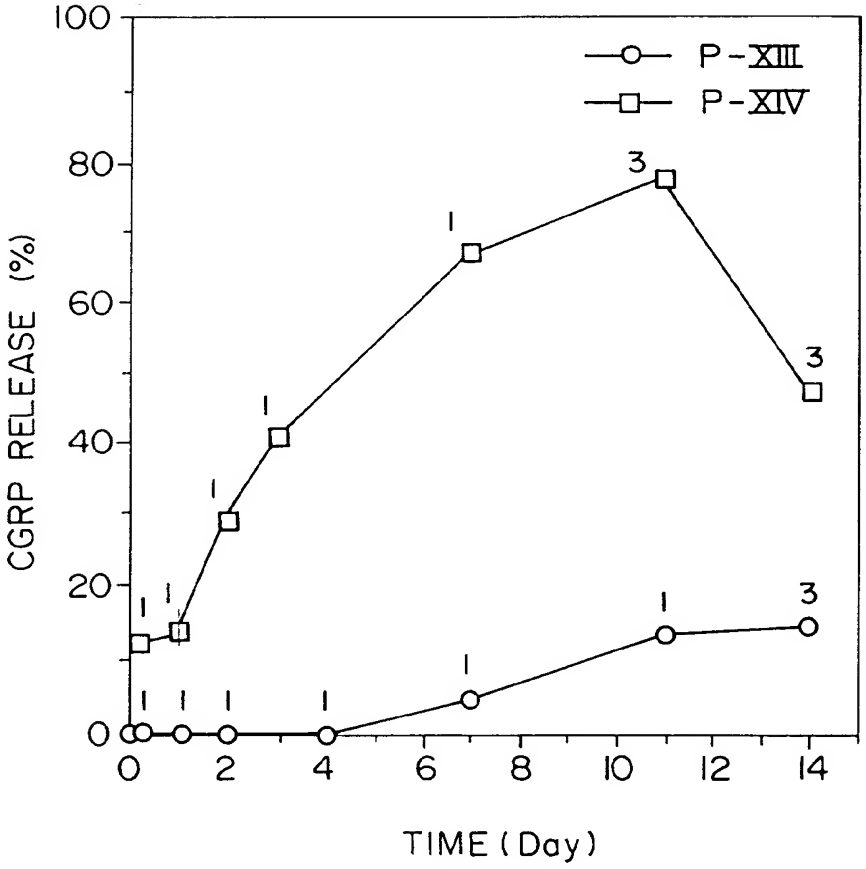
100
80
60
40
20
0
CGRP RELEASE (%)
P-XIII
P-XIV
0
2
4
6
8
10
12
14
TIME (Day)

FIG. 4

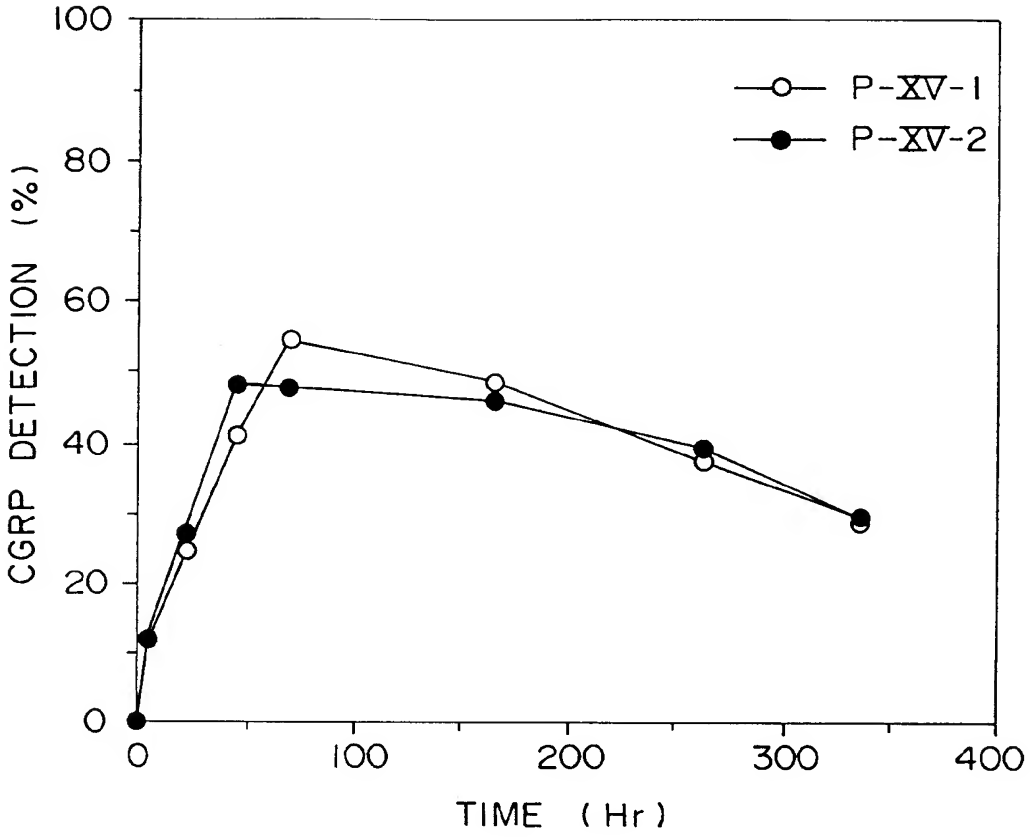
100
80
60
40
20
0
CGRP DETECTION (%)
0
100
200
300
400
TIME (Hr)
P-XV-1
P-XV-2

FIG. 5

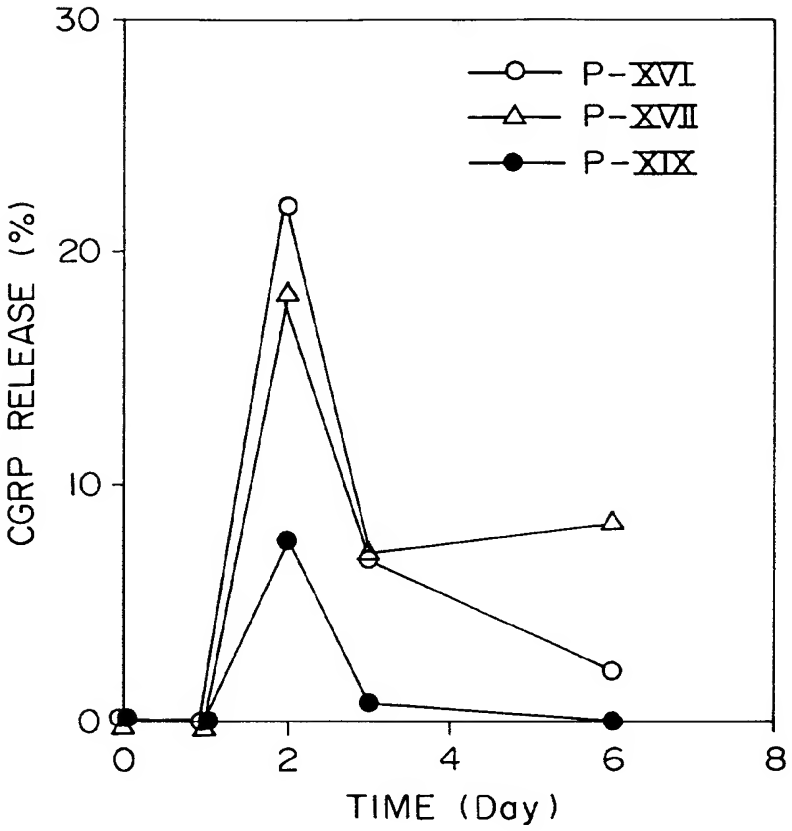

FIG. 6

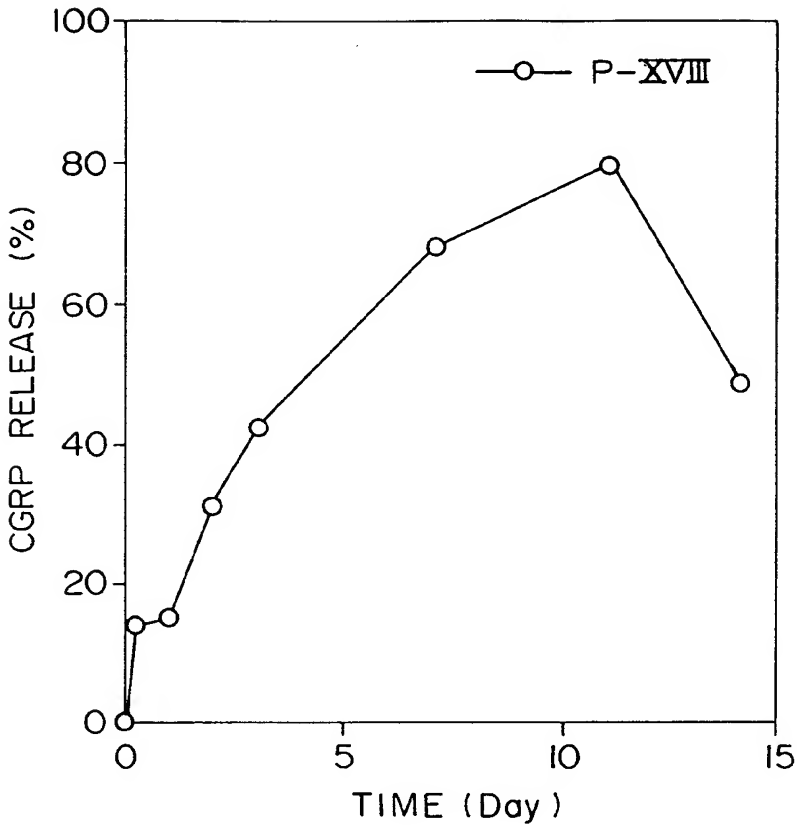

FIG. 7

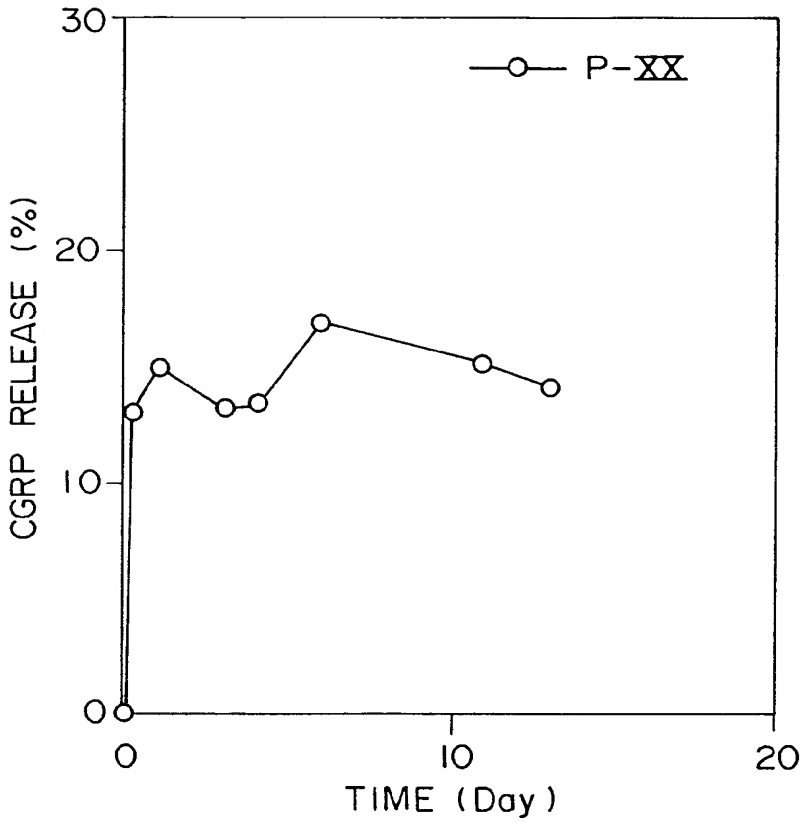

FIG. 8

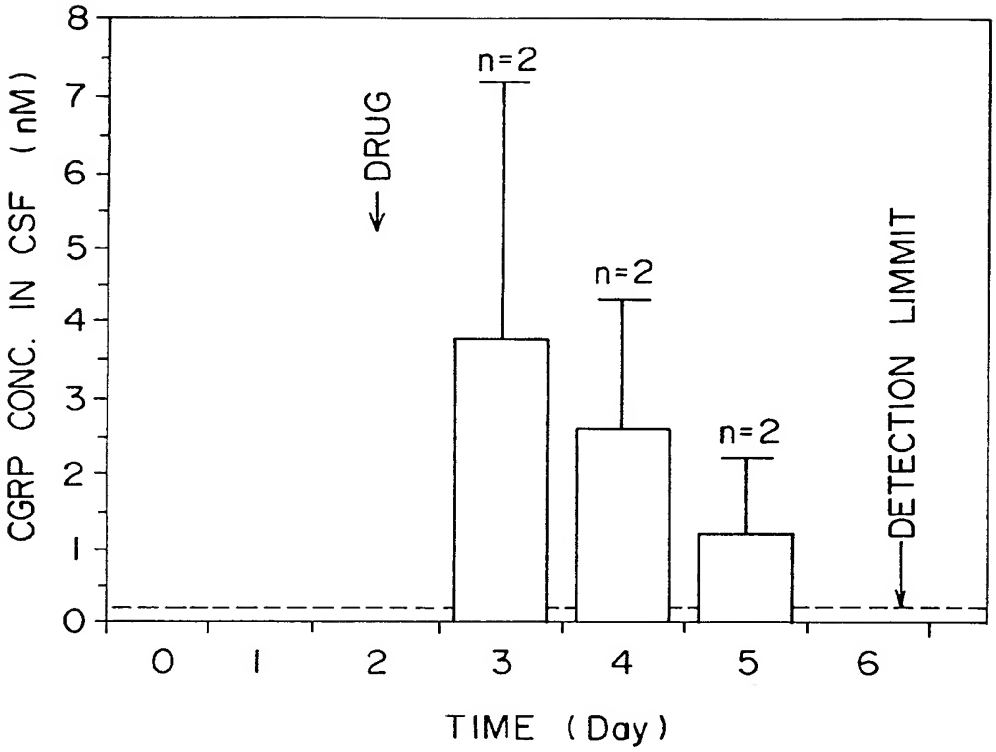
CGRP CONC. IN CSF (nM)
8
7
6
5
4
3
2
1
0
← DRUG
n=2
n=2
n=2
DETECTION LIMMIT
0
1
2
3
4
5
6
TIME (Day)

FIG. 9

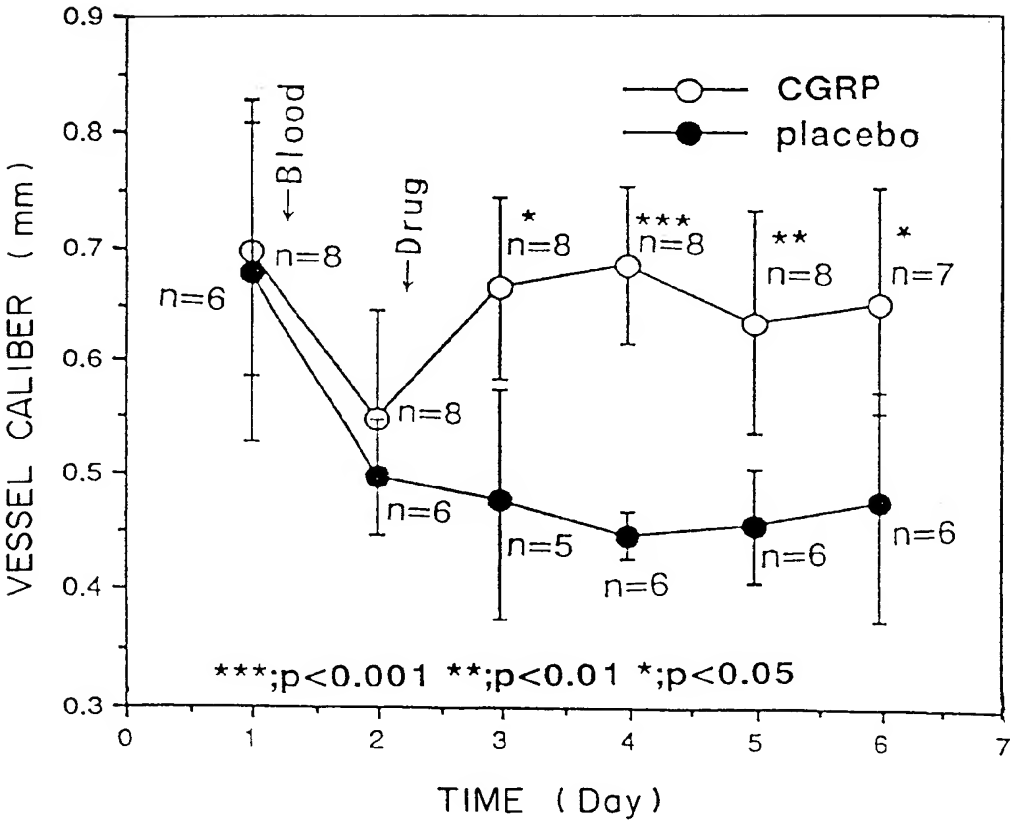
CGRP
placebo
Blood
Drug
VESSEL CALIBER (mm)
TIME (Day)
***;p<0.001 **;p<0.01 *;p<0.05
n=6
n=8
n=8
n=6
n=5
n=6
n=8
n=8
n=8
n=6
n=7
n=6
*
***
**
*

FIG. 10

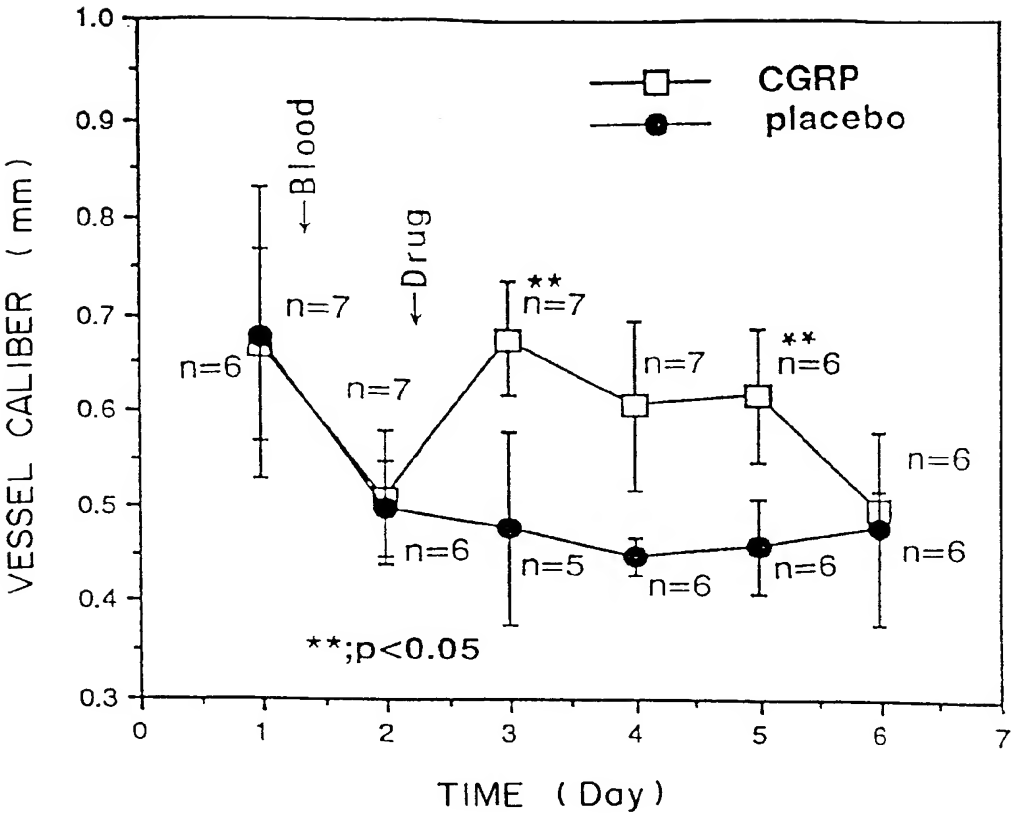
CGRP
placebo
Blood
Drug
VESSEL CALIBER (mm)
TIME (Day)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0
1
2
3
4
5
6
7
n=6
n=7
n=7
n=7
n=7
n=6
n=6
n=6
n=5
n=6
n=6
n=6
**
**
**;p<0.05

FIG. 11

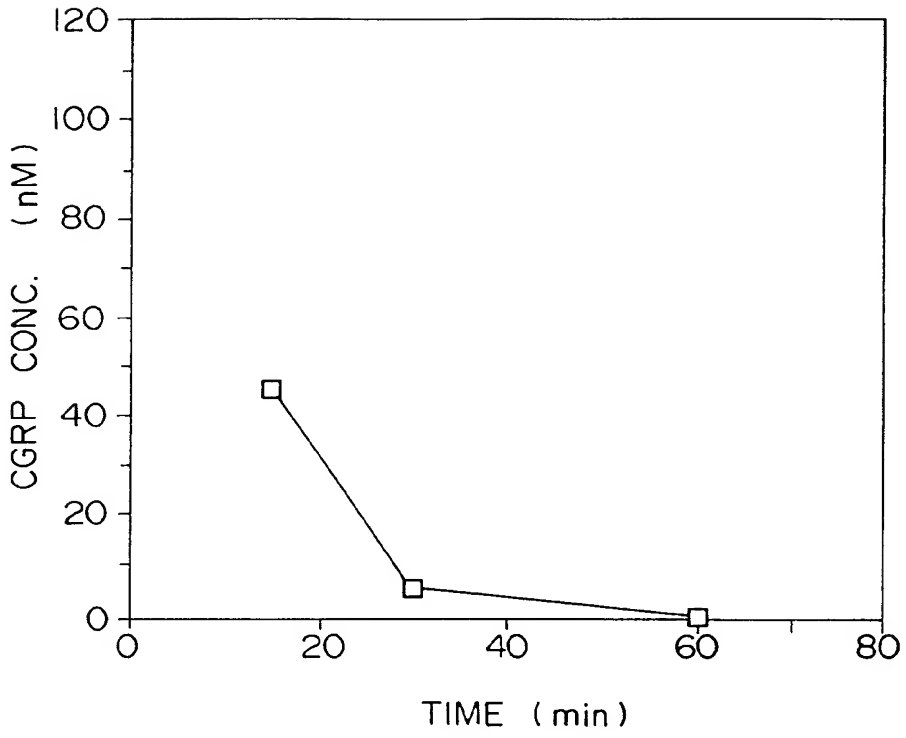

FIG. 12

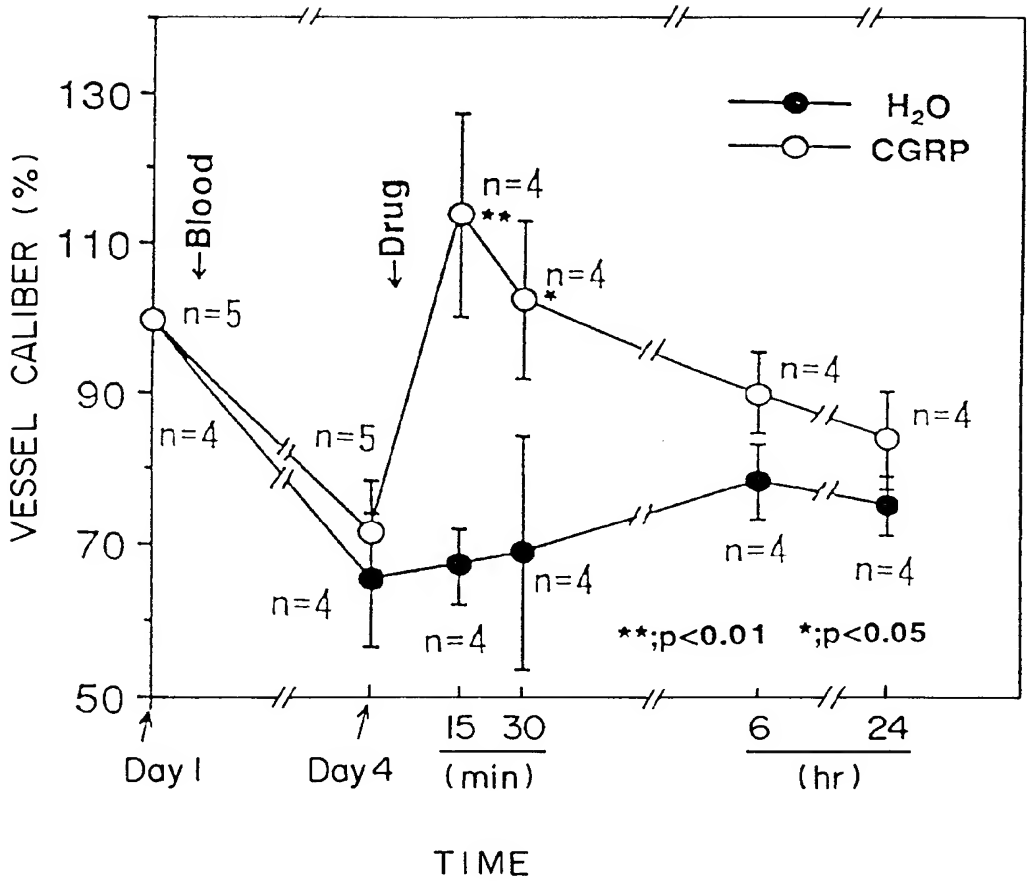
VESSEL CALIBER (%)
130
110
90
70
50
←Blood
←Drug
H_2O
CGRP
n=5
n=4
n=5
n=4
n=4
n=4
n=4
n=4
n=4
n=4
n=4
**
*
**;p<0.01 *;p<0.05
Day 1
Day 4
15 30
(min)
6 24
(hr)
TIME

FIG. 13

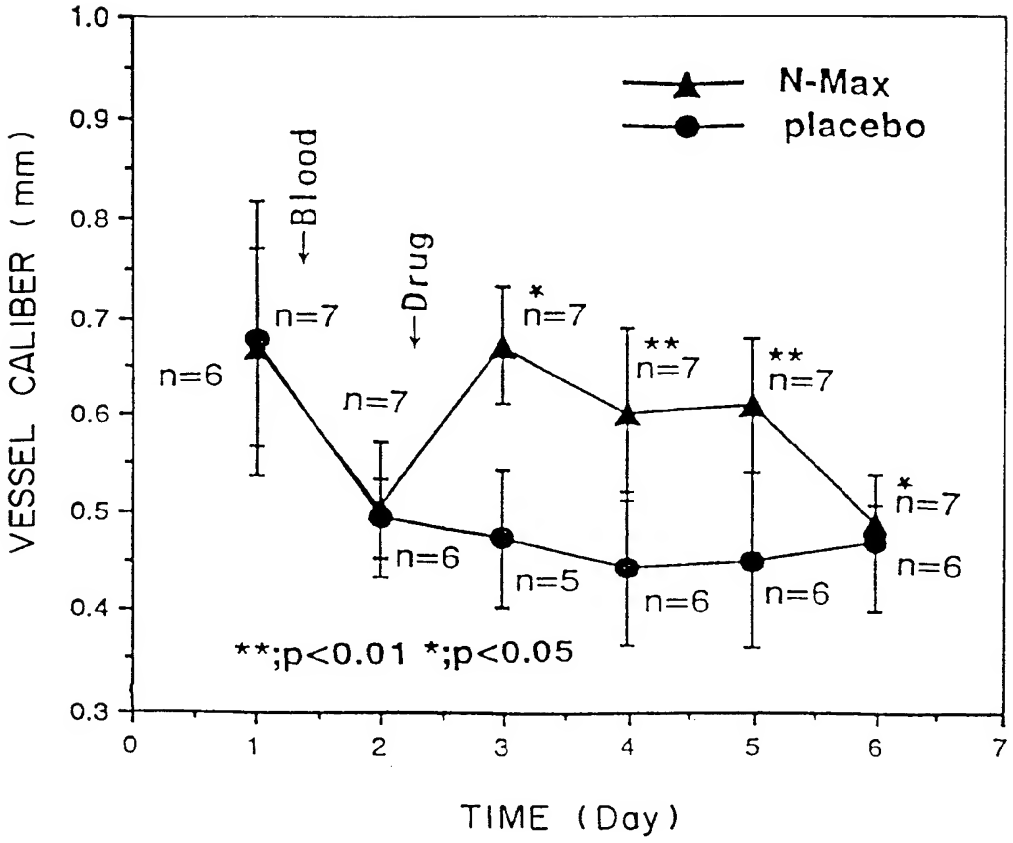
N-Max
placebo
VESSEL CALIBER (mm)
TIME (Day)
Blood
Drug
n=6
n=7
n=7
n=7
n=7
n=7
n=6
n=5
n=6
n=6
n=6
n=7
**;p<0.01 *;p<0.05
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0
1
2
3
4
5
6
7

FIG. 14

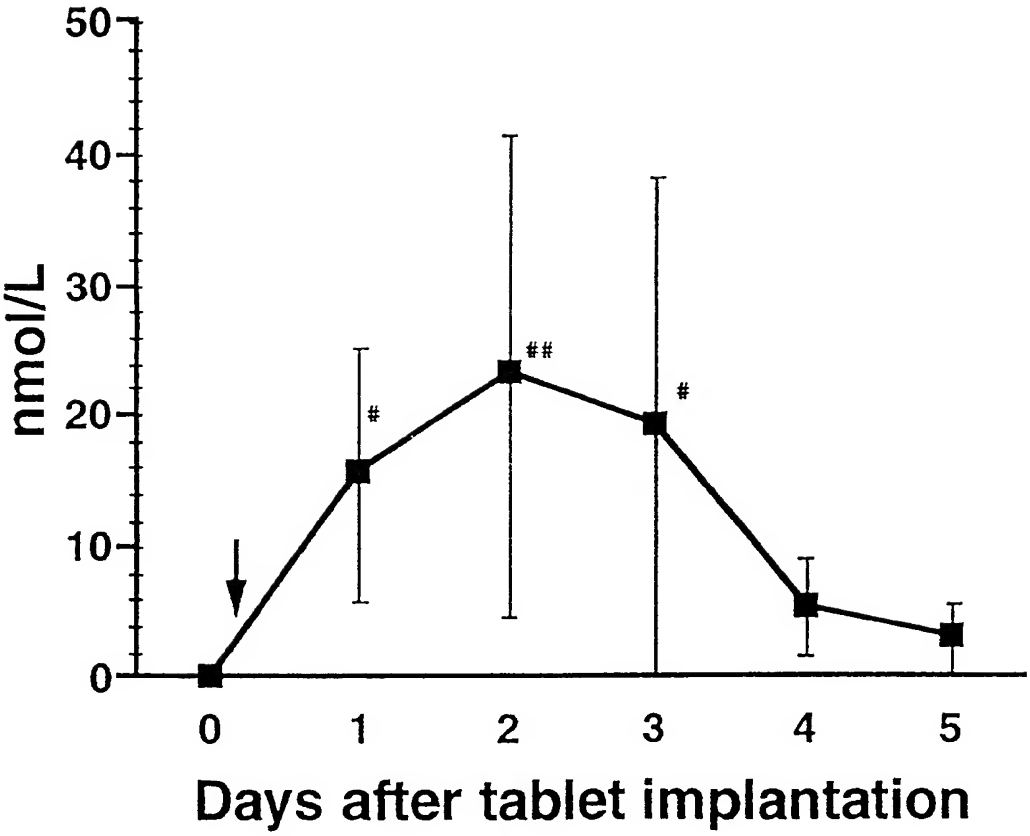

FIG. 15

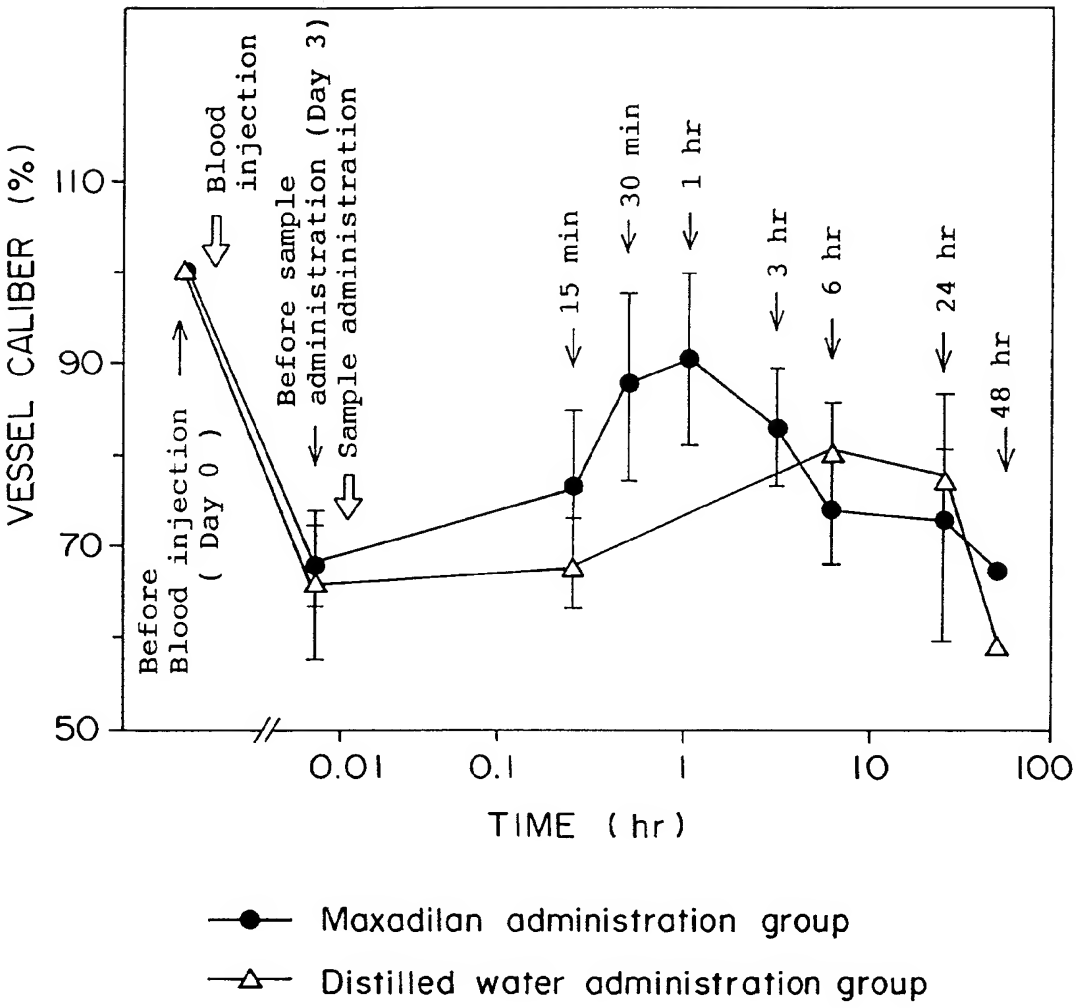
VESSEL CALIBER (%)
110
90
70
50
0.01
0.1
1
10
100
TIME (hr)
Blood injection
Before sample administration (Day 3)
Sample administration
Before Blood injection (Day 0)
15 min
30 min
1 hr
3 hr
6 hr
24 hr
48 hr
Maxadilan administration group
Distilled water administration group

FIG. 16

(19) Europäisches Patentamt
European Patent Office
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27.01.1994 JP 36254/94
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(54) Physiologically active substance-prolonged releasing-type pharmaceutical preparation

(57) A prolonged releasing pharmaceutical preparation is provided carrying a physiologically active substance, particularly, calcitonin gene-related peptide (CGRP) or a maxadilan (MAX). This pharmaceutical preparation can attain the expected effects by incorporating the physiologically active substance into a combination, as carriers for the physiologically active substance, of a cellulosic polymer and at least one auxiliary component selected from the group consisting of fats and oils, waxes, fatty acids, saccharides and polyacrylate ester derivatives. The pharmaceutical preparation can conveniently be used, in living bodies, particularly as an intrathecal implantation-type preparation.

EP 0 645 136 A3

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 94 11 4768

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
X	EP-A-0 196 700 (RIJKSUNIVERSITEIT GRONINGEN) 8 October 1986 * claims * ---	1	A61K9/22 A61K9/20 A61K38/22 C07K14/435
X	EP-A-0 255 404 (APS RESEARCH LTD) 3 February 1988 * claims * ---	1,6	
X	EP-A-0 211 266 (AMERICAN CYNAMID CO.) 25 February 1987 * example 14 * ---	1,2	
A	WO-A-89 03686 (CELLTECH LTD.) 5 May 1989 ---		
A	EP-A-0 309 100 (AMYLIN CORP.) 29 March 1989 ---		
A	J. BIOL. CHEM., vol. 266, no. 17, 1991 pages 11234-11236, E.A. LERNER ET AL. 'Isolation of maxadilan, a potent vasodilatory peptide from the salivary glands of the sand fly Lutzomyia longipalpis.' ---		TECHNICAL FIELDS SEARCHED (Int.Cl.6) A61K C07K
A	J. BIOL. CHEM., vol. 267, no. 2, 1992 pages 1062-1066, XP 000562629 E.A. LERNER ET AL. 'Maxadilan. Cloning and functional expression of the gene encoding this potent vasodilator peptide.' -----		

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	21 February 1996	Klaver, T

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

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(54) Delayed drug-releasing microspheres

(57) Microspheres prepared by emulsifying an aqueous phase containing a drug and an oil phase containing a biodegradable polymer to form a W/O emulsion, and then mixing and agitating this emulsion with another aqueous phase constituting a continuous phase to form a W/O/W emulsion. These microspheres can release a major portion of the drug after a predetermined period of time has elapsed after their administration to the patient, and are especially suitable for intracerebral implantation.

EP 0 765 659 A1

Description

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to microspheres capable of releasing a drug after a predetermined period of time. More particularly, it relates to microspheres which are adapted for intracerebral implantation having the purpose of suppressing cerebral vasospasm.

2. Description of the Prior Art

As microcapsules or microspheres used for the purpose of releasing a drug gradually over a long period of time, a great variety of pharmaceutical preparations made by supporting drugs on various carriers and polymeric matrices have been proposed.

Typical processes for the production of such pharmaceutical preparations include, for example, a process involving the formation of a W/O/W emulsion as described in Japanese Patent Laid-Open No. 201816/'87. Moreover, processes developed with a view to overcoming the disadvantages of such production process (e.g., a low degree of drug incorporation into the polymeric matrix, and a premature burst of the drug from microspheres) have also be proposed (see Japanese Patent Laid-Open Nos. 145046/'94 and 32732/'94).

However, these pharmaceutical preparations have the common purpose of releasing the drug steadily from the carrier over a long period of time, and successful examples of such release have been disclosed.

On the other hand, prolonged drug-releasing type pharmaceutical preparations which are to be implanted in particular organs for the purpose of treating special diseases have also be proposed. As a specific example of such pharmaceutical preparations, Japanese Patent Application No. 50953/'94 discloses a pharmaceutical preparation which uses a complex of a water-soluble polymer, an oil, a fatty acid and the like as the carrier and serves to suppress cerebral vasospasm, and also suggests that the timing of drug release can be controlled.

In order to effectively prevent or treat certain diseases as described above, including cerebral vasospasm accompanying abnormal contractions of cerebral blood vessels observed after a definite period of time has elapsed after the rupture of a cerebral aneurysm, it is important to release the drug with controlled timing instead of releasing it simply in a steady manner. For example, the above- described cerebral vasospasm comprehends early spasm observed within 24 hours after the rupture of a cerebral aneurysm and delayed spasm observed 4 days to 2 weeks after that, and the latter is considered to be of particular interest from a clinical point of view.

Under these circumstances, it would be meaningful to provide a pharmaceutical preparation in which the timing of drug release can be controlled as described in Japanese Patent Application No. 50953/'94, i.e., a major portion of the drug can be concentratively released after the lapse of a predetermined period of time (which preparation will be referred to herein as a "delayed drug-releasing type" pharmaceutical preparation).

Although delayed drug-releasing type pharmaceutical preparations have already been provided as described above, it is still desired to provide a further improved pharmaceutical preparation or drug delivery system having such a mode of drug release. Accordingly, an object of the present invention is to provide a drug delivery system of the delayed releasing type which can release the drug concentratively after the lapse of a predetermined period of time, instead of releasing it steadily.

SUMMARY OF THE INVENTION

The present inventors have carried on investigations on the development of a drug delivery system of the delayed releasing type by using, as the drug-carrying matrix, an α-hydroxycarboxylic acid polymer which has a good affinity for living bodies and produces little side effect. As a result, it has unexpectedly been found that, although such a polymer itself shows a parabolic degradation pattern in vivo, microspheres prepared by the so-called submerged drying W/O/W process involving the formation of a W/O emulsion by the emulsification of an oil phase containing a specific biodegradable α-hydroxycarboxylic acid polymer and an aqueous phase containing a drug exhibit the above-described delayed drug-releasing behavior.

Thus, as a means for solving the above-described problem, the present invention provides delayed drug-releasing microspheres prepared by a process comprising

(A) the step of emulsifying an aqueous solution containing a drug and a water-immiscible solution containing a biodegradable α-hydroxycarboxylic acid polymer to form a W/O emulsion, and
(B) the step of emulsifying the emulsion resulting from step (A) in an aqueous solution to form a W/O/W emulsion.

In one preferred embodiment, the present invention provides such microspheres adapted to cerebral implantation and, in particular, such microspheres adapted to cerebral implantation for the purpose of suppressing cerebral vasospasm.

Moreover, the present invention also provides a method for the prophylaxis or treatment of cerebral vasospasm by administering the above-described microspheres to a patient subject to the disease and, in particular, by implanting the above-described microspheres in the brain of the patient.

In a further embodiment, the present invention also provides the use of the above-described microspheres for the making of a pharmaceutical preparation useful in the suppression of cerebral vasospasm.

The microspheres of the present invention have a unique effect in that, even if the α-hydroxycarboxylic acid polymer itself shows a parabolic degradation pattern in the living body, they begin to release a required amount of the drug after the lapse of a predetermined period of time, without entailing a premature burst of the drug.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 includes electron micrographs of microspheres in accordance with the present invention, showing the particle structures of several fractions having different particle diameters;

Fig. 2 is a graph showing the in vitro drug-releasing behavior of microspheres in accordance with the present invention (particle diameter: greater than 150 μm);

Fig. 3 is a graph showing the in vitro drug-releasing behavior (in two runs) of microspheres in accordance with the present invention (particle diameter: 32 to 150 μm);

Fig. 4 is a graph showing the in vitro drug-releasing behavior (in two runs) of microspheres in accordance with the present invention (average particle diameter: less than 32 μm);

Fig. 5 is a graph showing the in vivo drug-releasing behavior of microspheres in accordance with the present invention; and

Fig. 6 is a graph showing the in vivo drug-releasing behavior of a tablet formed from microspheres in accordance with the present invention.

DETAILED DESCRIPTION OF THE INVENTION

The aqueous solution containing a drug, which is used to prepare microspheres in accordance with the present invention, can be prepared simply by dissolving the drug in sterilized purified water. In order to improve the solution stability of the drug, this aqueous solution may further contain a buffer agent (e.g., a phosphate buffer or a citrate buffer), osmoregulators (e.g., saccharides such as glucose, lactose, mannitol and sorbitol, and inorganic salts such as sodium chloride), and water-soluble polymers such as gelatin, alginine and gum arabic.

The drug contained in the aqueous solution can be any of various physiologically active substances, provided that they are suited to the objects of the present invention. Specific examples of the drugs intended for use in the present invention include adrenaline, abscisic acid, arginine vasotocin, angiotensinogen, angiotensin, angiotensin I converting enzyme, succus gastricus-inhibiting polypeptides, insulin, insulin-like growth factors, S-factor, erythropoietin, luteinizing hormone, luteinizing hormone-releasing hormone, progestogens, oxytocin, 2-octyl-γ-bromoacetoacetate, autacoids, gastrin, gastrin secretion-accelerating peptide, gastron, activated vitamin D_3, kallidin, calcitonin, calcitonin gene-related peptide (CGRP), maxadilans, kininogen, thymus hormone, glucagon, glucocorticoids, vasoactive intestinal peptide, plasma kallikrein, serum factor, blood glucose-elevating hormone, thyroid-stimulating hormone, thyrotropin-releasing hormone, thyroid hormone, melanophore-stimulating hormone, melanophore-stimulating hormone-releasing hormone, melanophore-stimulating hormone release-inhibiting hormone, corticotropin-like middle lobe peptide, urokinase, cholecystokinin octapeptide, cholecystokinin tetrapeptide, cholecystokinin variant, cholecystokinin-12, cholecystokinin pancreothymine, cholecystokinin, growth factor, substance P, female sex hormones, adipokinin, chorionic gonadotropin, nerve growth factor, pancreatic polypeptides, gonad-stimulating substance, gonadotropic hormones, growth hormone, growth hormone-releasing factor, secretin, caerulein, serotonin, fibroblast growth factor, kallikrein glandularis, somatostatin, somatomedins A and B, placental lactogen, thymosin, thymopoietin, thyroglobulin, traumatic acid, endothelial cell growth factor, mollusc heart stimulant neuropeptide, neurotensin, equine serum gonadotropic hormone, brain hormones, noradrenaline, vasopressin, estrogenic hormone, histamine, epidermic cell growth factor, parathyroid hormone, parathyroid-stimulating hormone, corticotropin-releasing factor, adrenocortical hormones, PACAP, bradykinin, bradykinin-like peptide, proinsulin, proopiomelanocortin, prostaglandins, pro PTH, prolactin, prolactin-releasing hormone, prolactin release-inhibiting hormone, florigene, human menopausal gonadotropin, bombesine, mineral corticoid, light adaptation hormone, methionyllysylbradykinin, 1-methyladrenaline, melatonin, motilin, androgen, diuretic hormone, lipotropin, renin, relaxin and follicle maturation hormone.

The drugs having the effect of suppressing cerebral vasospasm, which are particularly intended for use in the present invention, include CGRP, maxadilans, deferoxamine, methylprednisolone, nicorandil, nicaraben, magnesium sulfate, actinomycin D, 21-aminosteroid, isoproterenol, tPA, urokinase, nimodipine, hydro-cortisone, nicardipine, nifedipine, diltiazem, dilazep, teprothid, AA 861, papaverine, OKY 1581, amyl nitrite, erythrityl tetranitrate, isosorbide dinitrate, nitroglycerin, pentaerythritol tetronitrate, VIP, vasopressin, bradykinin, PACAP, SOD, catalase, bepridil, nadololol, felodipine, isradipine, verapamil, atenolol, metoprolol and propranolol.

Among these drugs, CGRP and maxadilans are deeply intended for use in the present invention. When these peptides (or proteins) are formed into microspheres in accordance with the present invention, they can be effectively used because, in cooperation with the biodegradability of the α-hydroxycarboxylic acid polymer used and the solubility of the drugs themselves in

body fluids, they exhibit unique drug-releasing behavior desired in the present invention. In this connection, the maxadilans which can be used in the present invention include proteins found in the salivary gland of the sand fly (Lutzomyia longipalpis) as described, for example, in E.A. Lerner, J. Biol. Chem., 267, 1062-1066 (1992), and analogous proteins as described in International Publication No. WO 95/04829.

The concentration of the drug in the aqueous solution cannot be clearly defined because it depends on the type of the drug used, and the type and severity of the disease to be treated. However, the drug should preferably be used at such a concentration as not to cause an excessive premature release (or burst) from the microspheres during treatment. By way of example, CGRP is usually used at a concentration of 1×10^{-8} to 100 mg/l and preferably 1×10^{-5} to 1 mg/l, though the present invention is not limited thereto.

The biodegradable α-hydroxycarboxylic acid polymers which can be used to form the polymeric matrix of microspheres in accordance with the present invention include, for example, homopolymers derived from one of glycolic acid, lactic acid, 2-hydroxybutyric acid, 2-hydroxyvaleric acid and 2-hydroxy-3-methylbutyric acid, as well as copolymers derived from two or more of the foregoing compounds. Among these polymers, homopolymers of lactic acid and copolymers of lactic acid and glycolic acid can conveniently be used because their characteristics as polymeric matrices for carrying drugs have been studied well and commercial products having various degrees of polymerization are available. The lactic acid used as a monomer may be its D- or L-isomer or a mixture containing its D- and L-isomers in any desired proportion, and polymers so prepared are commercially available.

In view of the objects of the present invention, when CGRP is chosen as the drug, it is especially preferable to use a copolymer prepared from a monomer mixture composed of L-lactic acid and glycolic acid in a molar ratio of 7:3 to 3:7 and having a molecular weight of about 4,000 to 11,000 as measured by gel permeation chromatography (GPC).

The water-immiscible solution containing a polymer as described above is usually prepared by dissolving the polymer in a halogenated hydrocarbon such as methylene chloride, chloroform or dichloroethane. However, methylene chloride is preferably used because it can be easily evaporated. The concentration of the polymer in this solution may generally be in the range of 10 to 100% by weight and preferably 50 to 80% by weight.

According to the present invention, the aqueous solution containing the above-described drug and the water-immiscible polymer solution are subjected to an emulsification treatment. This treatment is preferably carried out under such conditions as to produce a W/O emulsion in which most of the oil droplets contained therein have an average diameter of about 0.5 to 5 µm. The reason for this is that excessively small oil droplets may not exhibit the desired delayed drug-releasing behavior, and excessively large oil droplets may have low stability.

When the emulsification treatment is carried out, for example, by the application of ultrasonic waves, the mixed system may be ultrasonicated under ice cooling for one to several minutes by means of a commercially available ultrasonic homogenizer [e.g., Sonifier-250 (manufactured by BRANSON)]. Mechanical stirring may be used in combination with the ultrasonication. In such a case, good results will be produced by performing the mechanical stirring at a speed of about 10,000 rpm or greater.

In this emulsification treatment, one or more additives selected from surfactants and water-soluble polymers which contribute to the stabilization of the W/O emulsion may be added to the mixed system. Usable surfactants include anionic surfactants such as sodium oleate, sodium stearate and sodium lauryl sulfate, and nonionic surfactants such as polyoxyethylene sorbitan fatty acid esters, and usable water-soluble polymers include polyvinyl alcohol, polyvinyl pyrrolidone, carboxymethylcellulose, hydroxypropylcellulose, gelatin and the like. These additives may be used at such a concentration as to give a 0.01 to 10%(w/w) aqueous solution.

Subsequently, the W/O emulsion prepared in the above-described manner is emulsified with an aqueous solution constituting an outer aqueous phase to form a W/O/W emulsion. It is especially preferable to add the above-described emulsion stabilizer to this outer aqueous phase at the above-defined concentration. When methylene chloride is used as the solvent for the polymer, the solvent can be evaporated under submerged conditions by carrying out the emulsification treatment at room temperature by vigorous stirring (e.g., at a speed of about 10,000 rpm or greater). Consequently, microspheres containing the drug in the polymer matrix are formed in the water. In order to promote the evaporation of the solvent, the above-described treatment may be carried out under reduced pressure.

If necessary, the microspheres may be stabilized by stirring the emulsion under milder conditions (e.g., at about 500 rpm) for several hours. Thereafter, the microspheres are separated by precipitation (or centrifugation) or filtration, washed, and then vacuum-dried or freeze-dried. Thus, there can be obtained microspheres in accordance with the present invention. In certain cases, these microspheres may be classified by sieving.

The microspheres thus obtained exhibit delayed drug-releasing behavior in body fluids or simulated body fluids [e.g., Hartmann's fluid (manufactured by Green Cross Corporation)], without entailing a premature burst of the drug. By way of example, mention is made of microspheres prepared in accordance with the present invention by using CGRP as the drug and a lactic acid-glycolic acid copolymer (composed of lactic acid and glycolic acid in a molar ratio of 7:3 to 3:7 and having a molecular weight of about 4,000 to 11,000 as measured by GPC) as the polymer. When they are suspended in a

simulated body fluid and maintained at 37°C, they exhibit a bulk release of the drug after several days to ten-odd days, and when they are implanted in the brain of an experimental animal, they exhibit a bulk release of the drug after 2 to 8 days. In order to achieve the above-described drug-releasing behavior, microspheres having diameters of about 30 to 300 μm are preferred.

Generally, this timing of drug release can be retarded by increasing the proportion of lactic acid used to prepare the polymer. Those skilled in the art will be able to prepare microspheres having a desired timing of drug release by repeating trial runs according to the type of the drug used.

Microspheres in accordance with the present invention can be directly used for intracerebral implantation or oral administration, or can be used in the form of an injectable suspension. They can also be used in other dosage forms according to the route of administration. For example, as a pharmaceutical preparation for use in intracerebral implantation, they may be formed into tablets having a size which does not interfere with intracerebral implantation (e.g., having a thickness of 5 mm or less and, in the case of cylindrical tablets, a diameter of 8 mm or less). Such tablets can be formed according to any technique known per se, using excipients such as lactose, crystalline cellulose and starch (e.g., corn starch), and other commonly used additives such as disintegrators (e.g., carboxymethylstarch sodium and carboxymethylcellulose calcium), binders (e.g., hydroxypropylcellulose, gum arabic, dextrin, carboxymethylcellulose, methylcellulose and polyvinyl pyrrolidone) and lubricants (e.g., talc, magnesium stearate and polyethylene glycol 6000).

According to the present invention, there are provided microspheres which can release a major portion of the drug after a predetermined period of time has elapsed after administration of the pharmaceutical preparation, without entailing a premature burst of the drug. Consequently, when microspheres in accordance with the present invention are prepared by using, for example, a drug having the effect of suppressing cerebral vasospasm, they can treat that disease conveniently. Moreover, there is also provided a pharmaceutical preparation which is adapted to intracerebral implantation and hence more effective in such treatment.

The present invention will be more clearly understood with reference to the following examples using CGRP as a drug. However, these examples are not to be construed to limit the scope of the invention.

Example 1: Preparation of microspheres

6 mg of CGRP Was dissolved in 0.5 ml of distilled water for injection use. On the other hand, 10 g of a copolymer derived from L-lactic acid and glycolic acid (in a molar ratio of 1:1) [having a molecular weight of about 4,500 as measured by GPC and corresponding to PLG 1600ML commercially available from Kokusan Chemical Co., Ltd.] was dissolved in 10 g of methylene chloride. After the solutions so prepared were mixed, the resulting mixture was agitated by means of an ultrasonic homogenizer [Sonifier-250 (manufactured by BRANSON)] for one minute under ice cooling to form a W/O emulsion.

This emulsion was added to 2,500 ml of a separately prepared 0.1%(w/w) aqueous solution of polyvinyl alcohol, and the resulting mixture was stirred at about 10,000 rpm [by means of a D-7801 homogenizer (manufactured by Ystral GmbH)] for 5 minutes under ice cooling (at about 15°C) to form a W/O/W emulsion. This emulsion was further stirred at about 500 rpm [by means of a Three-One Motor (manufactured by Shinto Scientific Co., Ltd.)] at room temperature (about 25°C) for 3 hours. After the resulting emulsion was centrifuged and the supernatant was discarded, the separated microspheres were washed with distilled water on a membrane filter (manufactured by Millipore Ltd.) having a pore diameter of 0.4 μm. The washed microspheres were placed in a vacuum dryer (at room temperature) and dried under reduced pressure for 48 hours. Thus, microspheres predominantly having diameters in the range of about 40 to 100 μm were obtained in a total amount of 5 g.

These microspheres (hereinafter referred to as "MS-16") contained 36 μg of CGRP per 60 mg of microspheres.

When these microspheres were classified by means of standard sieves made of stainless steel, a fraction having an average particle diameter of greater than 150 μm [hereinafter referred to as MS-16(L)], a fraction having an average particle diameter of 150 to 32 μm [hereinafter referred to as MS-16(M)], and a fraction having an average particle diameter of less than 32 μm [hereinafter referred to as MS-16(S)] comprised 15%, 53% and 32%, respectively. Electron micrographs of these fractions are shown together in Fig. 1.

Example 2: Formation of a tablet from microspheres

60 mg of MS-16 obtained in Example 1 was intimately blended with 1.8 mg of hydroxypropylcellulose and 0.6 mg of magnesium stearate. Using a Correct N-30E single-shot tableting machine (manufactured by Okada Seiko Co., Ltd.), the resulting blend was pressed into a tablet (6 mm∅ x 2 mm).

Drug release tests

(1) In vitro test

Tubes having a capacity of 15 ml were sterilely charged with 5 ml of Hartmann's solution (manufactured by Green Cross Corporation). Then, 60 mg each of MS-16(L), MS-16(M) and MS-16(S) obtained in Example 1 were separately added to the tubes under sterile conditions. While these tubes were allowed to stand at 37°C, the solution was sampled from each tube at time 0 and

appropriate times between the 1st and 14th days, and analyzed by high-performance liquid chromatography (HPLC) to determine the amount of CGRP released into Hartmann's solution. The amount of CGRP released was plotted as a function of time to obtain graphs showing the respective drug-releasing patterns. These graphs are given in Figs. 2, 3 and 4.

The conditions employed for HPLC were as follows:

Mobile phase:

Liquid A:	A 0.1% aqueous solution of trifluoroacetic acid (TFA).
Liquid B:	A 0.085% solution of TFA in CH_3CN. (A concentration gradient was created in such a way that the proportion of liquid B increased from 20% to 60% over a period of 20 minutes.)
Column:	Capsule Pack C_8SG300 (manufactured by Shiseido Co., Ltd.; 6 mm∅ x 35 mm).
Flow rate:	1.5 l/min.
Detection:	214 nm.

It can been from the respective figures that, in particular, the microspheres having diameters of 32 μm or greater released CGRP from 10 or 11 days after the start of the test, without entailing a premature burst of the drug.

(2) In vivo test
(Implantation of a pharmaceutical preparation in the brain)

Five rabbits (weighing 2.5 to 3.0 kg) were anesthetized with pentobarbital (50 mg/kg for each animal) and fixed in a prostrate position. An incision was made in the head to expose the occipital periosteum (or dura mater), and the occipital bone was drilled to expose more of the dura mater. Thereafter, an about 8 mm incision was made in the dura mater and the arachnoidea, and a pharmaceutical preparation was implanted therethrough. For this purpose, 60 mg of MS-16 obtained in Example 1 was used in two of the five animals, one tablet obtained in Example 2 in other two animals, and one placebo tablet (not containing CGRP) in the remaining one animal. After the dura mater, muscles and skin were sutured, an appropriate dose of an antibiotic was administered to the site of incision.

Cerebrospinal fluid was collected daily from the aforesaid rabbits according to the following collection method, and the CGRP concentration (in nM) in the cerebrospinal fluid was measured according the following measuring method.

(Method for the collection of cerebrospinal fluid)

The above rabbits were anesthetized with pentobarbital and fixed in a prostrate position. An incision was made in the head to expose the occipital periosteum (or dura mater). Then, an incision was made in the occipital periosteum and cerebrospinal fluid (CSF) was collected through this incision.

(Method for the measurement of the CGRP concentration in cerebrospinal fluid)

This method comprises a radioimmunoassay procedure as described below.

Measuring tubes were charged with 4000 cpm of a labeled compound [2-(^{125}I-iodohistidy^{10}) CGRP]. Separately, 100 μl each of 1, 2, 5, 10, 50, 100, 500 and 1000 fmol standard solutions were prepared by using synthetic CGRP (manufactured by Bachem) as a standard substance. To the tubes containing 100 μl of a test sample, a standard solution or water were added 100 μl of an antibody solution [prepared by dissolving RPN 1841 (manufactured by Amersham) in 2 ml and diluting the solution to 12.5 ml] and 600 μl of an analytical buffer solution [50 mM sodium phosphate (pH 7.4), 0.3% bovine serum albumin, 10 mM EDTA]. These tubes were covered and allowed to stand at 4°C for 5 days. Then, 250 μl of a dextran/charcoal solution [50 mM sodium phosphate (pH 7.4), 0.25% gelatin, 10 mM EDTA] was added to the tubes, which were immediately centrifuged at 2000 x g for 20 minutes. Using a gamma counter, both the precipitate and the supernatant were counted for 200 seconds. Then, the concentration of the physiologically active substance (CGRP) in CSF was determined by reference to a standard curve constructed with the solutions of the standard substance. The results obtained with MS-16 and tablets are shown in Figs. 5 and 6, respectively.

It can be seen from these figures that, when MS-16 or a tablet formed therefrom is implanted in the brain of animals, it releases a major portion of CGRP concentratively after a predetermined period of time has elapsed after its implantation.

Claims

1. Delayed drug-releasing microspheres which can be prepared by a process comprising

   (A) the step of emulsifying an aqueous solution containing a drug and a water-immiscible solution containing a biodegradable α-hydroxycarboxylic acid polymer to form a W/O emulsion, and
   (B) the step of emulsifying the emulsion resulting from step (A) in an aqueous solution to form a W/O/W emulsion.

2. Microspheres as claimed in claim 1 which are adapted to intracerebral implantation.

3. Microspheres as claimed in claim 2 which are adapted to intracerebral implantation for the purpose of suppressing cerebral vasospasm.

4. Microspheres as claimed in claim 3 wherein the drug is a peptide having the effect of suppressing cerebral vasospasm.

5. Microspheres as claimed in claim 4 wherein the peptide is selected from the group consisting of calcitonin gene-related peptide and maxadilans.

6. Microspheres as claimed in any one of claims 1 to 5 wherein the biodegradable α-hydroxycarboxylic acid polymer is a polymer of lactic acid or a copolymer of lactic acid and glycolic acid.

7. Microspheres as claimed in any one of claims 1 to 6 wherein the formation of an emulsion in step (A) is carried out by ultrasonication.

8. Use of microspheres for the making of a pharmaceutical preparation useful in the suppression of cerebral vasospasm, wherein the microspheres are delayed drug-releasing microspheres prepared by a process comprising

   (A) the step of emulsifying an aqueous solution containing a drug and a water-immiscible solution containing a biodegradable α-hydroxycarboxylic acid polymer to form a W/O emulsion, and
   (B) the step of emulsifying the emulsion resulting from step (A) in an aqueous solution to form a W/O/W emulsion.

9. Use as claimed in claim 8 wherein the pharmaceutical preparation is adapted to intracerebral implantation.

10. Use as claimed in claim 8 or 9 wherein the drug is selected from the group consisting of calcitonin gene-related peptide and maxadilans.

Fig. 1

MS−16
(L)

MS−16
(M)

MS−16
(S)

FIG. 2

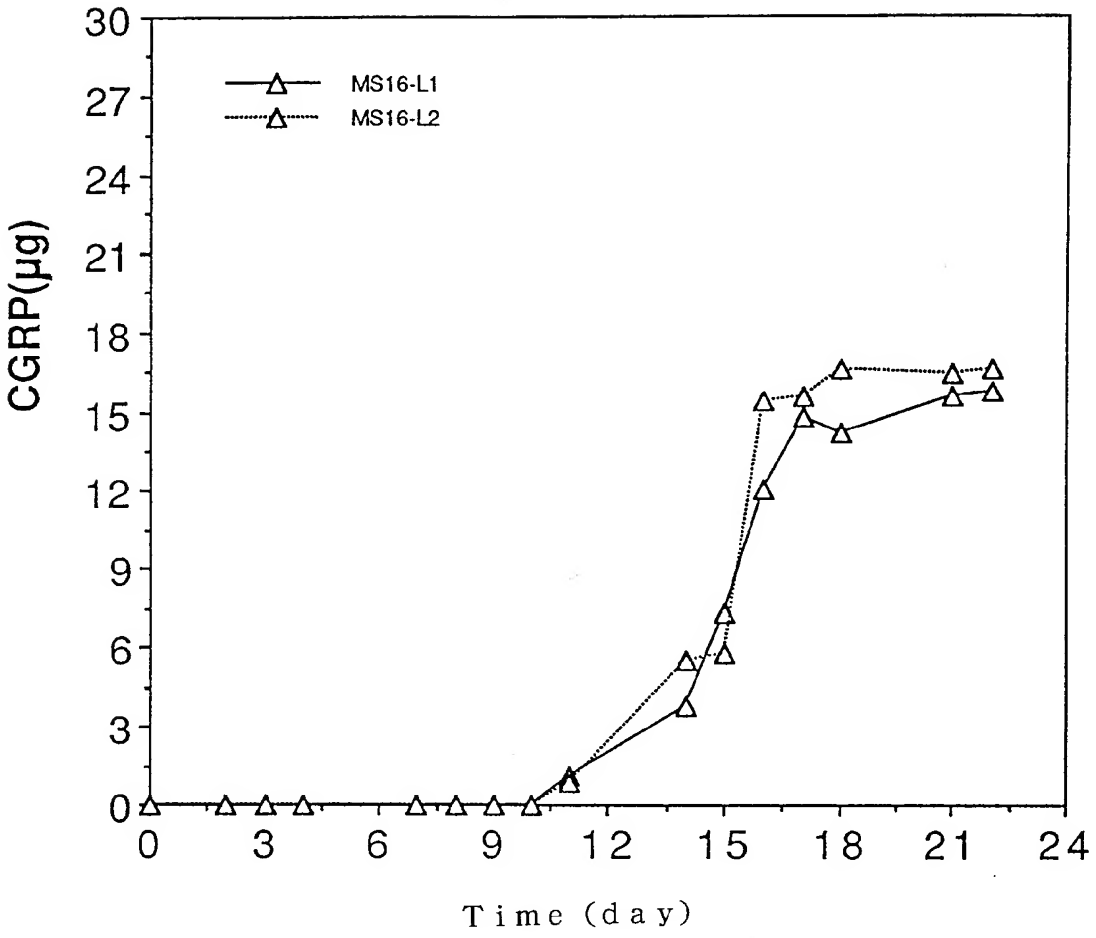

30
27
24
21
18
15
12
9
6
3
0
CGRP(μg)
MS16-L1
MS16-L2
0
3
6
9
12
15
18
21
24
Time (day)

FIG. 3

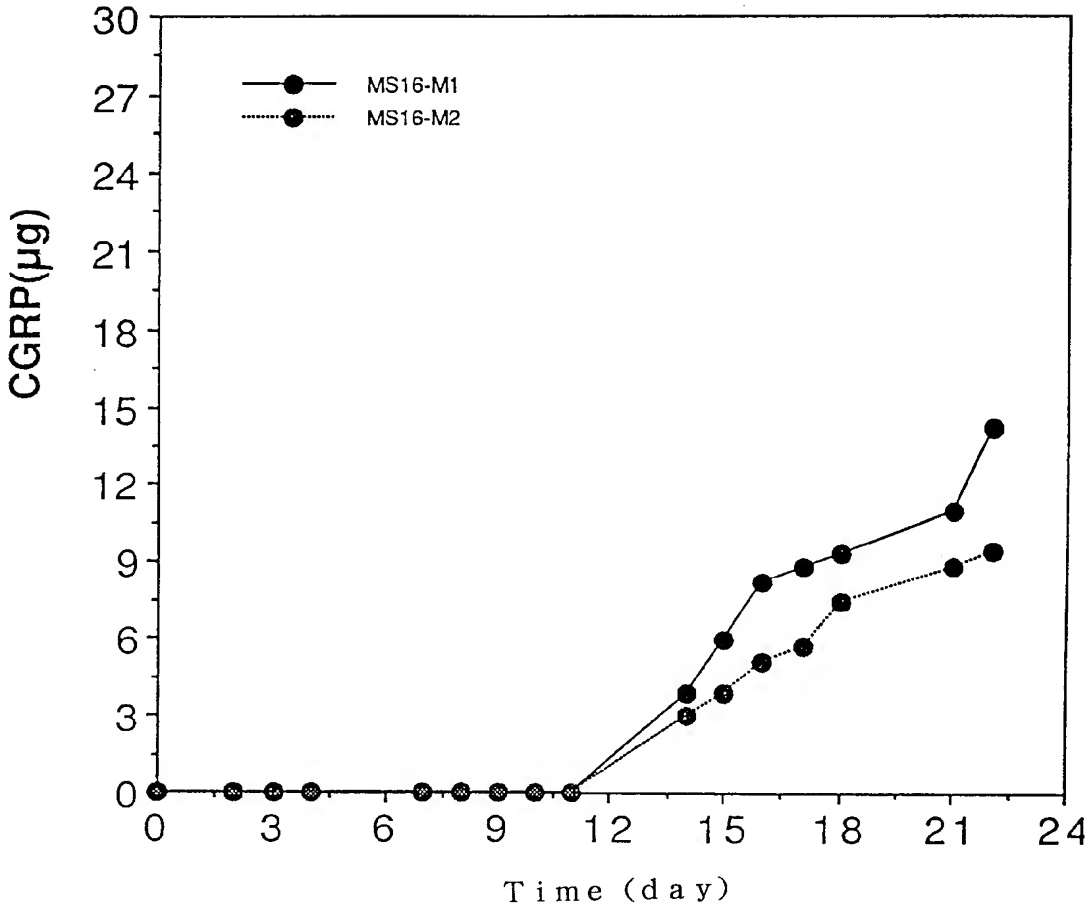

30
27
24
21
18
15
12
9
6
3
0
CGRP(μg)
MS16-M1
MS16-M2
0
3
6
9
12
15
18
21
24
Time (day)

FIG. 4

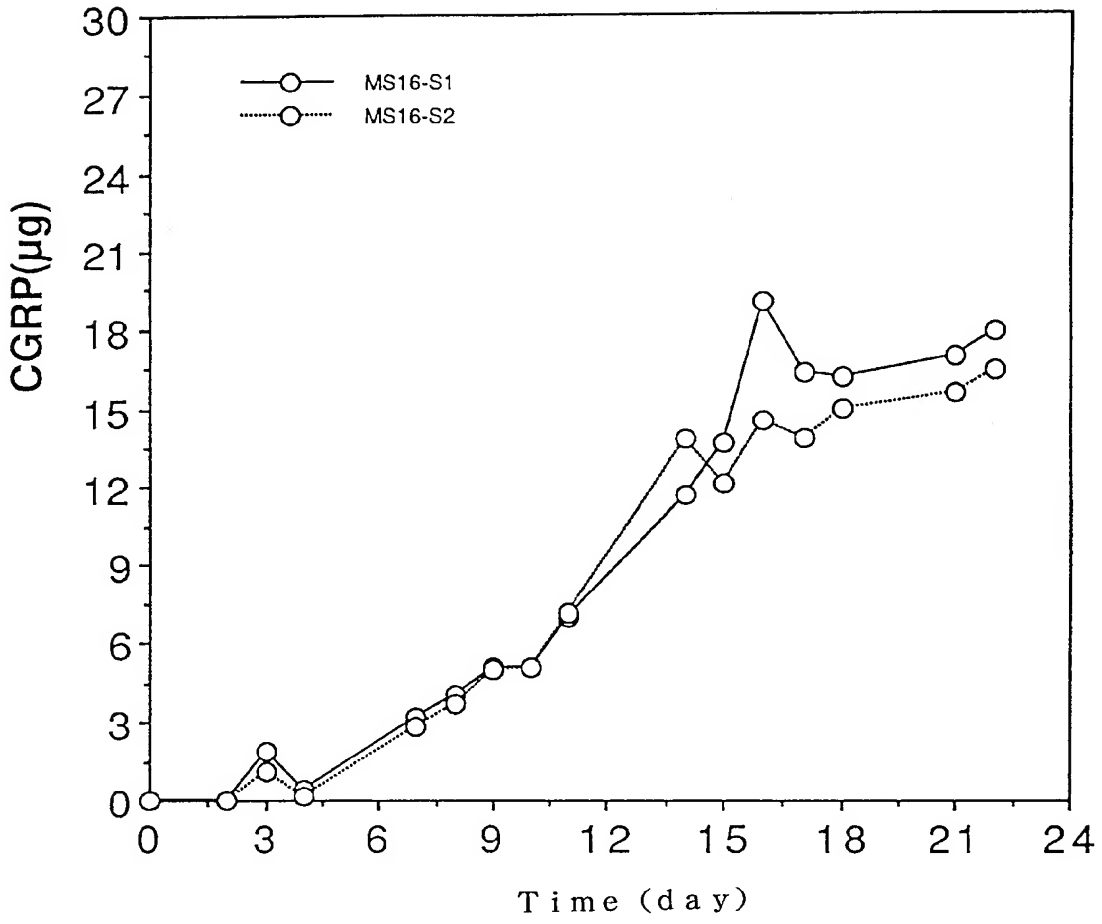


FIG. 5

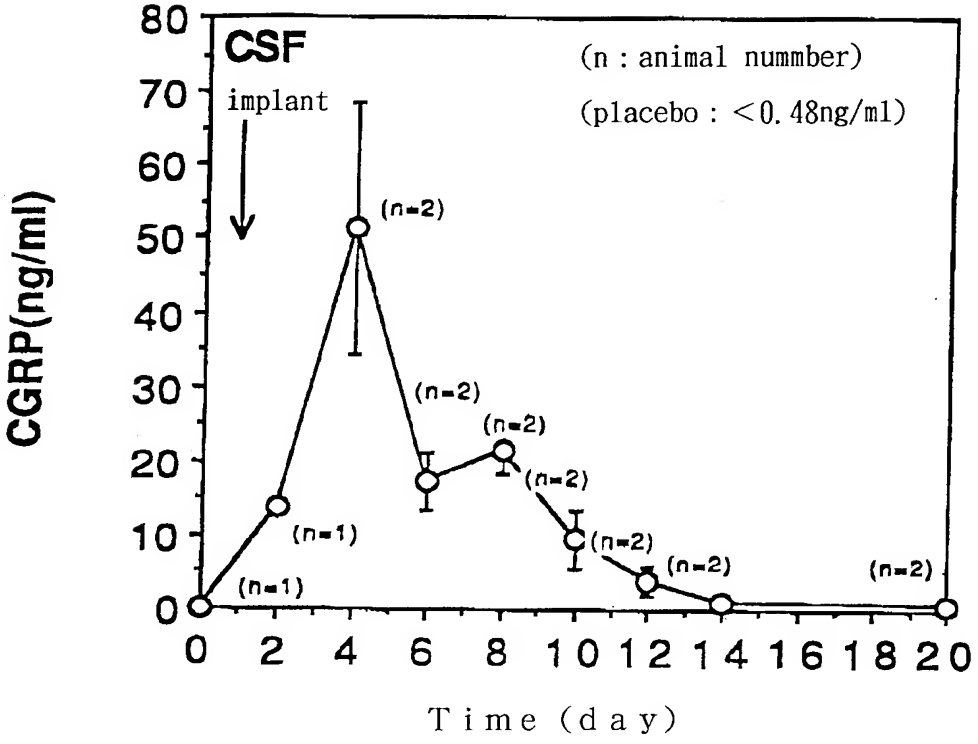
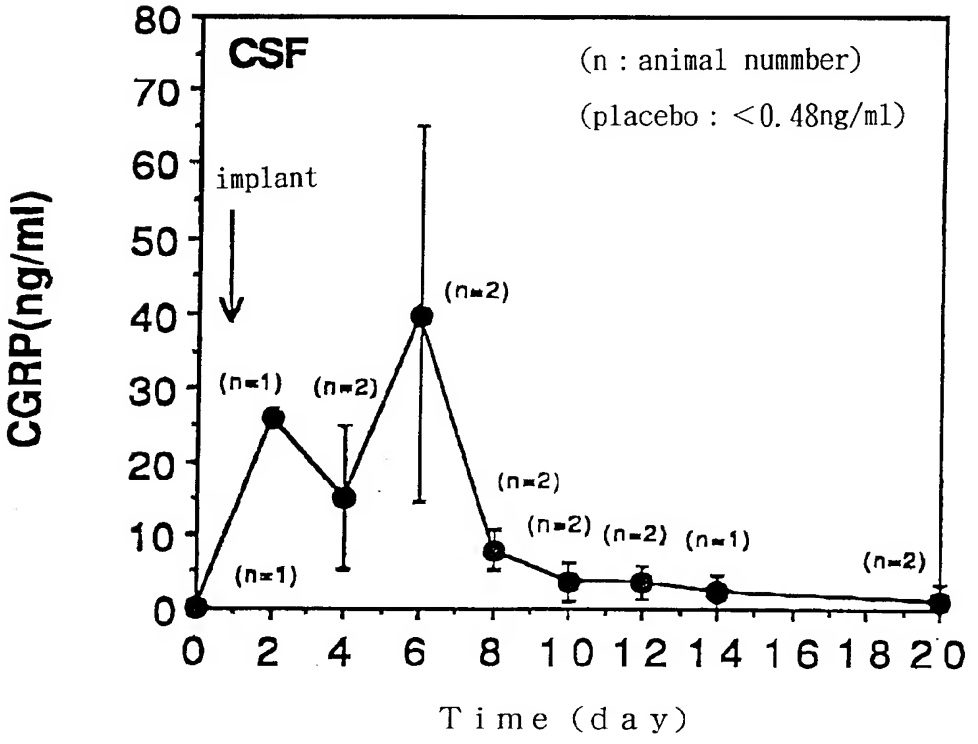

CSF
(n : animal nummber)
(placebo : <0.48ng/ml)
implant
CGRP(ng/ml)
80
70
60
50
40
30
20
10
0
(n=1)
(n=1)
(n=2)
(n=2)
(n=2)
(n=2)
(n=2)
(n=2)
(n=2)
0 2 4 6 8 10 12 14 16 18 20
Time (day)

FIG. 6


CSF
(n : animal nummber)
(placebo : <0.48ng/ml)
implant
CGRP(ng/ml)
80
70
60
50
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(n=2)
(n=2)
(n=2)
(n=1)
(n=2)
0 2 4 6 8 10 12 14 16 18 20
Time (day)

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 96 11 4888

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
X	EP-A-0 330 180 (BIOMATERIALS UNIVERSE, INC.)	1-3,6-8	A61K9/113 A61K9/16
Y	* page 3, line 10 - line 56 * * page 4, line 46 - page 5, line 57 * ---	4,5,9,10	
X	WO-A-91 12882 (MEDGENIX GROUP S.A.)	1-3,6-8	
Y	* page 9, line 15 - page 10, line 3 * * page 4, line 23 - page 6, line 22 * ---	4,5,9,10	
X	JOURNAL OF CONTROLLED RELEASE, vol. 17, no. 1, 1991, pages 23-32, XP000223264 H.T.WANG ET AL.: "Influence of formulation methods on the in vitro controlled release of protein from poly(ester) microspheres"	1-3,6-8	
Y	* abstract * * page 24, right-hand column, paragraph 4 * * page 30, left-hand column * ---	4,5,9,10	
X	JOURNAL OF MICROENCAPSULATION, vol. 12, no. 1, 1995, pages 59-69, XP000486815 H.K.SAH ET AL.: "Biodegradable microcapsules prepared by a w/o/w technique: effects of shear force to make a primary w/o emulsion on their morphology and protein release"	1-3,6-8	TECHNICAL FIELDS SEARCHED (Int.Cl.6) A61K
Y	* abstract * * page 68, paragraph 2 * ---	4,5,9,10	
D,X	EP-A-0 582 459 (TAKEDA CHEMICAL INDUSTRIES, LTD.)	1-3,6-8	
Y	* column 2, line 19 - line 33 * * column 3 - column 10 * ---	4,5,9,10	
	-/--		

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
MUNICH	10 January 1997	Tzschoppe, D

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons
.....
& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C01)

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 96 11 4888

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
D,Y	EP-A-0 645 136 (SHISEIDEO COMPANY LIMITED) * abstract * ---	4,5,9,10	
Y	EP-A-0 292 710 (AMERICAN CYANAMID COMPANY) * abstract * * column 4, line 43 - line 44 * -----	4,5,9,10	
			TECHNICAL FIELDS SEARCHED (Int.Cl.6)

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EPO FORM 1503 03.82 (P04C01)

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(54) Liposomal human calcitonin gene-related peptide composition and preparation of the same

(57) The present invention provides a pharmaceutical composition of hCGRP and the preparation of the same. The composition comprises liposomes from natural soybean phospholipid, in which the weight ratio of hCGRP to soybean phospholipid is 1-2 to 100-8000. The half-life of the composition is longer than 72 hours, and the stability of the composition is also elongated. The composition can be administrated by intravenous infusion, oral, nasal mucosal spray in an amount of 0.1-10 pg hCGRP per kg body weigh to treat hypertension and congestive heart failure of a human. The bioavailability of approximately 80%.

EP 0 845 269 A2

Description

Background of The Invention

This invention relates to liposomal complex of human calcitonin gene-related peptide (liposomal hCGRP) composition and the preparation of the same, in particular to a product obtained by combining phospholipid and hCGRP.

Human α-type calcitonin gene-related peptide (hCGRP) is an endogenous neuromodulator and is known as the most potent vasodilator up to now. Its marktable product can be purchased in the world. However, hCGRP, as shown in other peptides, is unstable in storage (*in vitro*, aqueous solution) and circulation (*in vivo*) with half-life of 9-12 min, and it is difficult to utilize such a peptide as a drug for clinical application.

In order to shed a new light on hCGRP as a clinical drug, this object of the invention is to provide a liposomal hCGRP and the preparation of the same, whereby a novel phospholipids are associated with hCGRP to obtain a very stable and effective product. This liposomal hCGRP can release hCGRP gradually from the liposome to achieve long-term effect with half-life of 72 min *in vivo*, which can be effective in preventing and curing cardiovascular diseases.

Description of The Invention

In order to realize this objective, the present invention relates to a pharmaceutical composition of hCGRP comprising liposomes prepared from natural soybean phospholipid. It is characterized by the fact that the weight ratio of hCGRP to soybean phospholipid is 1-2 to 100-8000(w/w), specially 1.5-2 to 2500-6000(w/w).

Said pharmaceutical composition of liposomal hCGRP containing 20-2000pg hCGRP in 5ml said composition is more preferred.

Mannitol, sorbitol, isotonic saline and dextrose or other pharmaceutical acceptable materials can be added in said pharmaceutical composition of liposomal hCGRP.

The present invention also relates to a method for preparing the pharmaceutical composition of the liposomal hCGRP characterized by following steps:

(1) adding sterilized and distilled water to purify and dry soybean phospholipid in a weight ratio of lipid to water being greater than 1 to 1000, followed by sonicating to obtain small and single-membrane vesicles of lipid bilayer;

(2) mixing hCGRP, dissolved in H_2O with a ratio of peptide to H_2O of 1 to 1000-25000, with the above soybean phospholipid in a ratio of the peptide to lipid being 1-2 to 100-8000, more particularly 1.5-2 to 2500-6000, sonificating and incubating at 37°C for 30-60 min to obtain a stable composition of liposomal hCGRP.

The composition of liposomal hCGRP thus obtained can be further lyophilized, and then dissolved in H_2O to obtain a aqueous solution containing 20-2000pg hCGRP per 5ml solution.

The present invention also relates to a method for treating hypertension and congestive heart failure of a human by administrating to the patient the aforementioned pharmaceutical composition of liposomal hCGRP. Said method includes intravenous infusion, oral, nasal mucosal spray. Among them, intravenous dose of liposomal hCGRP is 0.1-10 pg hCGRP per kg body weigh. The bioavailability of liposomal hCGRP is approximately 80%.

Detail illustration is shown in the following.

Theoretical basis of preparation of liposomal complex of hCGRP

Composition and sequence of the amino acids of hCGRP are characterized by (1) 8 of 37 amino acids of hCGRP are polar amino acids, with hydrophilic side chains, and 16 of 37 amino acids are apolar with hydrophobic side chains; (2) 4 of 8 polar amino acids are basic amino acids with positive charges in H_2O, and pH of hCGRP is 10. One molecule hCGRP contains 2 arginine, 2 lysine (Lys) and 1 aspartic acid (Asp). Arg and Lys are charged positively, Asp is charged negatively at physiological pH. hCGRP in which the ratio $(\Sigma Lys + \Sigma Arg)/(\Sigma Glu + \Sigma Asp)=4$, is a very strong basic peptide. In physiological pH hCGRP is charged by net 3 positive charges. hCGRP contains 16 hydrophobic amino acids and 6 hydrophilic amino acids, and is a very typical amphoteric molecule.

By analysis of phospholipid composition, specially soybean phospholipid, it has been ascertained that (1) acidic lipids with negative charges in head groups in H_2O are greater than 40% of total phospholipid, (2)unsaturated fatty acid in soybean phosphglycerides are approx 70%, with protection effect from oxidation and hydrolyzation, (3) at the limit of very low lipid concentration (lipid:H_2O<1:100 w/w), the thermodynamically stable state is the dispersion of single walled vesicles of soybean phospholipid bilayers. The vesicle size range is 20-50 nm.

In the present invention by thin layer chromatography and gas-phase chromatography the soybean phospholipid components were analyzed, corrected and quantified using a standard phospholipids (Sigma). The soybean phosphol-

ipid used for clinical injection contains 44.9% acidic phospholipids including phosphatidylserine (17.2%), phosphatidylglycerol (8.1%), phosphatidylinositol (15.2%) and cardiolipin (4.4%) with rich negative charges in H_2O, and linoleic acid (58.31%), palmitic acid (24.36%), linolenic acid (7.32%), oleic acid (5.9%) and stearic acid (3.88%) with 71.53% unsaturated fatty acid and 28.47% saturated fatty acid. Liposome structure, as noted earlier, are formed spontaneously in H_2O by phospholipid molecules, from many different phospholipids, and the composition most frequently used has been the natural phospholipid extracted from cell membrane, such as soybean phospholipid [Imperial Chemical Industrial Ltd. and National Research Development Corporation, British Patent 1523965 1977]. Small single membrane liposomes range in diameter from approx. 200Å to 500Å, and consist of a single lipid bimolecular layer surrounding an aqueous compartment. Small single membrane liposome is characterized by (1) osmotic insensitivity (2) about 70% of the total lipid is located in the outer leaflet of the vesicle (3) by the very low lipid concentration limit with the thermodynamically stable state being the single membrane vesicles of lipid bilayer [Gruler H. Microstructure and transport properties of single shelled vesicles and monolayers of lipid mixtures and lipid/protein alloyes, in Liposomes Drugs and Immunocompetent Cell Functions. Edited by Claude Nicolau 1981.99 15-27] (4) medium to large liposomes (MLV and LUV) are cleared rapidly from circulation after i.v. administration, while small unilamellar liposomes offer the potential for sustained drug release in blood stream and targeting to tissues other than the reticuloendothelial cell. Great emphasis is placed upon liposome as a biomembrane model, creating the possibility of *in vivo* application in medicine and research [Yang F.Y., The Application of Liposome in The Research in Biomembrane and Pharmacology, *SHENWUHUAXUE YU SHENGWUWULI JINZHAN (Biochemistry And Biophysics,* 1977 6:36]. Soybean phospholipid is a novel lipid existing in biomembrane, which have been used for the preparation of artificial membranes, such as liposomes [Biomembrane Group, Institute of Biophysics, Chinese Academy of Sciences, *SHENWUHUAXUE YU SHENGWUWULI JINZHAN (Biochemistry And Biophysics,* 1978 4:1].

By sonication and incubation the possibility presents itself for the polar interaction of the negatively charged group of the phospholipid with the positive charged groups of the amino acid residues of hCGRP on the outer surface of the membrane through ionic bonding with water. The apolar groups are located in the hydrophobic area of the membrane as a result of hydrophobic force, which includes the tails of the phospholipid and the hydrophobic amino acid residues from the hCGRP while the thermodynamic stability of liposomal hCGRP has been achieved, with an increase of half-life from 9-12 to 72 min and long-term storage in aqueous solution for two years (original storage time of 15 days). The effective dose of only 10^{-5} of liposomal hCGRP represents remarkable reduction in dosage and furthermore it can be absorbed by mucosal administration including oral, nasal and rectum mucosa with bioavailability of approximately 80%. Clinical studies indicated that remarkable treatment effect of liposomal hCGRP on 200 patients with hypertension and congestive heart failure had been achieved, and no secondary effect had been observed.

Example 1

Preparation of liposome from soybean phospholipid

25g soybean phospholipid was rotary evaporated (by using rotary evaporator, XZ-6, produced by Zhongkeyuan Kelong Corp.) from a chloroform:methanol (2:1,v/v) solution to form a thin golden film on the walls of a 1000 ml round bottomed flask.

Alter the last obvious traces of solvent had been removed, rotary evaporating was continued for 15 min, followed by drying for a further 15 min under a nitrogen atmosphere. The lipid was suspended in 250ml of distilled water and shaken with a few glass beads using a shaker (HZS-D, produced by Harbin Donglian Corp.) and then by an ultrasound bath machine (DF-6P3c, produced by Ningbo Xinyi Research Institute) for 30 min.

Reconstitution of hCGRP in liposome membrane

10 mg hCGRP was dissolved in 250 ml destilled water, and stirred for 5min. hCGRP solution was mixed with the above liposome solution (250ml), stirred for 5 min, and sonicated for 2-3min, three times with interval of 3-5 min (by using an ultrasound bath machine, DF-6P3c, produced by Ningbo Xinyi Research Institute). Then the mixture was incubated at 37°C for 40 min (by using an ultrasound bath machine, produced by Harbin Donglian Corp.).

Sedimentation of liposomal hCGRP

The reconstituted solution was sedimented by Ultracentrifuge (400000×g, for 40min, at 4°C, VAC 602, WEB Leipzig, Germany) and washed with distilled water three times.

Lyophilization and Resolution

The sedimented liposomal hCGRP was lyophilized by using lyophilizer LGJ (produced by Instrument Plant of Academy of Military Medical Sciences (China)) and dissolved in distilled water (passed through 6# sterilizing filter) (lipid:H_2O=1:1000w/w), the above solution was sterilized at 100°C for 30 min, and enclosed.

Example 2

The procedure of Example 1 was repeated using various ratios of peptide to lipid(w/w). hCGRP reconstitution in membrane, the reconstitution efficiency and the stability of the final product were compared, as shown in Table 1.

Table 1

Effect of Liposomal hCGRP with various ratios of peptide to lipid (w/w) in preparative procedure for the reconstitution efficiency and vasodilatory activity.		
Ratio of hCGRP to lipid (w/w)	Free hCGRP (%)	Relative Vasodilatory Activity (%)
1:1	80.2±10.1%	0.02±0.01%
1:10	24.5±3.6%	1.2±0.3%
1:100	15.7±1.9%	32.6±6.7%
1:1000	0.1±0.02%	95.1±11.4%
1:10000	0.1±0.03%	94.9±13.9%
1:250000000	0±0%	100±0%

Method: liposomal hCGRP was prepared according to the procedure of Example 1 with various ratios of peptide to lipid (w/w), followed by centrifugating and determining of hCGRP content in supernatant liquid as free hCGRP not reconstituted in the membrane. After that each group of samples were divided into two groups, one was stored in -70°C condition after being sterilized and sealed as control, and another was dissolved in H_2O with the ratio of lipid to H_2O being 1 to 1000(w/w) stored at room temperature after sterilized and stored under nitrogen. Alter storage time of 24 months vasodilatory activity of samples was measured, shown as % of control. Each date is mean±SD of five independent data.

In Table 1 it is seen that when the ratio of lipid to peptide was above 1000(w/w), hCGRP had been reconstituted in membrane with little free hCGRP, and vasodilatory activity remained at above 95% after 24-month-storage.

Example 3

In the following test, a similar procedure to that of Example 1 was repeated with the exception of using various ionic strength to study the effect of ionic strength in solution on reconstitution efficiency of hCGRP in lipid membrane. Table 2 list the effect of ionic strength on hCGRP reconstitution.

Table 2

the effect of ionic strength in solution on reconstitution efficiency of hCGRP in lipid membrane.					
NaCl Conc. (mM)	0	10	50	100	150
Free hCGRP	0.2±0.1%	11.6±1.8%	24.5±4.2%	35.5±7.9%	36.1±4.1%

Method: In an NaCl aqueous solution with different concentrations, hCGRP was reconstituted in soybean phospholipid membrane, followed by centrifugating and determination of free hCGRP content in supernatant liquid, shown as % of total hCGRP. The ratio of peptide to lipid was 1 to 1000(w/w), each date was mean ± SD of five independent experimental data.

In Table 2, it is indicated that reconstitution efficiency decreased with the increasing ionic strength of the solution.

The purified H_2O solution may offer a favorable environment for interaction and association between lipid and peptide.

Comparative Example 1

The same procedure of Example 1 was repeated with the exception of using two non-charged lipids instead of soybean phospholipid.

By determination of free hCGRP content in the supernatant liquid after centrifugation and chromatographic analysis, it was shown that the reconstitution efficiency of hCGRP in soybean phospholipid membrane achieved 99.9%, but in PC and PE membrane were only 21.2% and 30.3% respectively, indicating that negative charge of phospholipid is very important for reconstituting hCGRP in membrane successfully.

Table 3

Comparison of reconstitution efficiency of hCGRP in soybean phospholipid (SP), phosphatidylcholine (PC), and phosphatidylethanolamine (PE) membrane(%).				
Sample	Kd	%	Kd	%
hCGRP	0.52	100%	0.04	0%
hCGRP+PC	0.52	79.8%	0.04	21.2%
hCGRP+PE	0.52	69.3%	0.04	30.3%
hCGRP+SP	0.52	0.1%	0.04	99.9%

Comparative Example 2

Three different phospholipid including soybean phospholipid (SP), phosphatidylcholine (PC) and phosphatidylethanolamin (PE) were used for liposomal hCGRP preparation, and the structure integrity of hCGRP in three liposome membranes was analyzed during 24-month storage by HPLC, as shown in Table 4.

Table 4

Effect of various phospholipids on integrity of hCGRP reconstituted in lipid membrane									
	time (months)								
	0	3	6	9	12	15	18	21	24
SP(% area)	98.4 ±2.4	98.4 ±2.1	99.0 ±1.9	98.0 ±2.4	97.8 ±2.1	97.8 ±2.0	97.1 ±1.8	97.1 ±1.9	97.0 ±1.7
PC(% area)	99.2 ±3.3	90.2 ±2.9	83.3 ±3.4	76.4 ±3.1	70.1 ±2.6	68.2 ±2.5	66.1 ±1.9	65.8 ±2.0	65.1 ±1.9
PE(% area)	98.9 ±2.9	94.5 ±3.1	89.2 ±2.2	85.3 ±3.3	82.1 ±1.9	76.6 ±1.7	70.3 ±1.6	68.5 ±2.1	64.4 ±2.2

Method: hCGRP in sample was extracted with acid solution and analyzed by reverse phase HPLC; retention time and peak area of hCGRP during chromatography was recorded. Each data is mean ±SD of five independent experimental data. hCGRP standard (BACHEM, Swizerland) was used for correction of retention time.

Results in Table 4 indicated that little change of hCGRP purity was observed during 24-month storage with decrease of 1.4%, indicating that hCGRP renconstituted in soybean phospholipid membrane is very stable during storage, but in PE and PC membranes are unstable and integrity of hCGRP remained only 64.4% and 65.1%.

Comparative Example 3

In the following test, phosphatidylcholine (PC) and phosphatidylethanolamin(PE) were used for liposome-hCGRP preparation and compared with soybean phospholipid liposomal hCGRP. During 24-month storage vasodilatory activities of three various liposomal hCGRP were determined and compared as shown in Table 5.

Table 5

Effect of various phospholipid on vasodilation of hCGRP reconstituted in lipid membrane (ED_{50} values × 10^{-8} mg/ml)									
Samples	storage (months)								
	0	3	6	9	12	15	18	21	24
Control (at -70°C, n=5)									
mean ± SD	4.7 ±0.6	4.9 ±0.5	5.2 ±0.7	5.1 ±0.8	5.3 ±0.5	5.2±0.7	5.1 ±0.6	5.2 ±0.6	5.3±0.7
PS in H_2O (at 25°C, n=5)									
mean ± SD	5.1 ±0.7	4.9 ±0.8	4.9 ±0.9	5.3 ±0.7	5.5 ±0.6	5.2±0.7	5.5 ±0.8	5.6 ±0.6	5.9±0.8
PC in H_2O (at 25°C, n=5)									
mean ± SD	4.9 ±0.8	15.2 ±3.2	50.6 ±10.3	151 ±21.2	451 ±99.3	865 ±153	1133 ±333	1566 ±439	1923 ±544
PE in H_2O (at 25°C, n=5)									
mean ± SD	5.2 ±0.7	10.3 ±2.4	33.4 ±6.5	99.1 ±15.8	329 ±82.8	634 ±101	903 ±125	1234 ±289	1633 ±345

Methods: Experiments were performed on New Zealand white rabbits (weight 2.5-3.5kg) that were anesthetized with pentobarbital sodium (30mg/kg, i.v.).

Rabbits were placed in headholer, diameters of ocular vessels (conjuctive) were measured by use of a microscope equipped with a TV camera coupled to a video monitor. Images were recorded in computer and vessel diameters were measured later with an image analyzer software. The analytical system of microcirculation was purchased from DAHENG Co. China. 10 μl of diluted samples was dropped into the eye of the rabbits and the images of ocular vessels were recorded in the computer.

During storage of 24 months, ED_{50} values ($\times 10^{-18}$ mg/ml) for vasodilation of liposomal hCGRP were determined. Little changes in soybean phospholipid membrane of liposomal hCGPR were observed. But, for PC and PE liposomal hCGRP, their ED_{50} decreased 100-1000 folds after 24-month storage, indicating negative charges in lipid membrane is very important for stable association between the peptide with lipid.

Experiment 1

Analysis of hCGRP-liposome complex

Methods: Liposomal hCGRP and free hCGRP were analyzed by the method of Berk [Berk D. and Marcinka K., Gel Chromatography in Separation Methods. Deylz ed. 1984, 271]. hCGRP samples reconstituted with or without soybean phospholipid (1.0 ml in 0.1M Tris-HCl, pH8.8) were applied to a 1.5x46cm sephadex G-50 fine column in 0.1M Tris-HCl, pH8.8. Blue dextran 2000 (Pharmacia) and $^{32}PO_4$ (England) were mixed with separated sample as mark of Vo and Vi respectively.

Free hCGRP in Sephadex G-50 fine was chromatographed at Kd=0.52, but liposomal hCGRP Kd=0.44, similar to blue dextron 2000, indicating that the liposome-hCGRP complex had been formed. The data obtained by gel filtration were described in Table 5.

Table 6

Determination of hCGRP binding with soybean phospholipid			
	Kd(G-50)		
hCGRP	0.52	0.54	0.51
hCGRP + Lipid	0.04	0.04	0.05

Table 6 (continued)

Determination of hCGRP binding with soybean phospholipid			
	Kd(G-50)		
Blue Dextran 2000	0		
$^{32}PO_4$	1		
Each data entry represents individual experiment result.			

As shown in Table 6, after the reconstitution of hCGRP with soybean phospholipid, much larger complex of hCGRP with lipid than hCGRP had been formed, indicating that the properties of hCGRP and soybean phospholipid give a new light on the preparation of a stable liposomal hCGRP.

Experiment 2

Analysis of physical and chemical stability of liposomal hCGRP

It is clear that any liposomal formulation must have adequate stability over the time period between its preparation and ultimate use so as to be a pharmaceutical carrier. The surface of liposome membrane, as mentioned above, has large amounts of negative charges, which prevents change of their size induced via the fusion between liposomes. In an environment of large amounts of water, the negative charged particles are in a thermodynamic stable state and large amount of unsaturated tails in phospholipid can reduce possibility of water molecule inserting themselves into lipid bilayer and thus preventing degradation of lipid and peptide molecules from auto-hydrolysis and auto-oxidation.

Phospholipids are subject to hydrolysis in aqueous media, resulting initially in the formation of the corresponding lysophospholipid and fatty acid. During the storage of our liposome or liposomal hCGRP, the contents of lyophospholipid were determinated as a criteria of chemical stability by TLC, while the size of liposome was measured by gel filtration observing the position of elution peak as a criteria of physical stability [Szoka F., et al., Comparative properties and methods of preparation of lipid vesicles (liposomes), Arm. Rev. Biophys. Bioeng. 1980 9:467.5].

Analysis LPC Content

Changes of LPC content in soybean phospholipid liposome. Storage conditions: at 25°C, samples in H_2O Lipid : H_2O=1:1000(w/w) and sterilized at 100°C for 30 min and sealed.

Methods: Analysis of LPC content in the liposome was done by TLC. Silica gel H (Type 60) from E. Merk in Germany. LPC standard was purchased from Sigma, as control of LPC in samples, and its Rf value is 0.04 in our experimental conditions. After samples were sterilized at 100°C for 30min, the chemical stability of liposome membrane was determined by analysis of LPC content at interval of 3 months during storage. Each data is mean ±SD of 5 independent TLCs. The mixture of the samples and standard LPC was used for corrective assay by single direction TLC and double direction TLC, indicating that LPC of the mixture was only one component on the silica gel.

During storage period of 24 months, the content of LPC was increased progressively from 2.1±0.34% to 4.7±0.51% ($p<0.01$) for soybean phospholipid vesicles and from 1.9±0.22% to 3.4±0.46% ($p<0.01$) for the liposomal hCGRP, respectively. The degradation percentages of phospholipid molecules were 2.6% and 1.5% for liposome and liposomal hCGRP respectively, indicating that the reconstituted hCGRP can increase stability of membrane by its positively charged groups which interact with negatively charged groups of phospholipid.

Determination of liposome size was carried out by means of gel filtration (Kd) during storage period. Kd values were unchanged either for soybean phospholipid liposome or for the reconstituted liposome with hCGRP, indicating that our liposomes are thermodynamically stable in 1000:1(H_2O: phospholipid, w/w) environment during 24 months storage after sterilization.

Experiment 3

Analysis of the stabilities of hCGRP reconstituted in liposome membrane

Stability of hCGRP reconstituted in liposome membrane was observed by (A) Sephadex G-50 fine gel filtration to measure the dissociation of hCGRP from the liposome; (B) microcirculation observation system to see the vasodilatory

activity, and compare with free hCGRP during storage period.

A. The stability of association of hCGRP with membrane (Table 8).

Methods: During storage period of the reconstituted liposome after being sterilized at 100°C for 30 min, free hCGRP, dissociated from the liposome, was determined by gel filtration at the interval of 3 months. The absorption at UV 206 run of hCGRP has been correlated with 0.52 of the distribution coefficient (Kd) in correction of hCGRP standard. At this Kd value, we can investigate whether hCGRP is dissociated from the reconstituted liposome during storage period. Sephadex G-50 fine gel filtration was carried out, with each data being mean±SD of 3 independent operations.

During the storage period of 24 months the hCGRP reconstituted in the liposome membrane was not dissociated into free hCGRP by observation of the 206nm absorption band of eluent solution at Kd=0.52. This result has shown us that hCGRP can form very stable complex with soybean phospholipid vesicles by our experimental procedure based on the characteristics of their molecular structure. All samples were stored at 25°C after sterilized and sealed. The ratio of lipid: H_2O is 1:1000 in the reconstituted liposome of soybean phospholipid.

B. Measurement of vasodilatory activities of hCGRP

hCGRP is an endogenous neuromodulator and most powerful vasodilatator known by us. We investigated vasodilatory activities of hCGRP reconstituted in the liposome membrane of soybean phospholipid and in comparison with free hCGRP in H_2O and human plasma during storage period.

Table 7

Comparison of vasodilation of hCGRP reconstituted in the liposome membrane of soybean phospholipid and free hCGRP.

A. in H_2O, 25°C

day	hCGRP				Liposome-hCGRP			
	0	30	60	90	0	30	60	90
1	+189	+142	+94	+82	+191	+189	+192	+201
2	+191	+133	+99	+79	+193	+194	+190	+191
3	+181	+152	+84	+63	+184	+186	+188	+181
4	+192	+161	+99	+88	+199	+202	+198	+200
5	+179	+165	+114	+102	+221	+219	+221	+209
mean ±SD	+187 (8.9)	+151 (14.2)	+98 (9.2)	+83 (12.4)	+198 (16.4)	+198 (13.2)	+198 (12.7)	+196 (14.3)

B. in plasma, 37°C

hour	hCGRP				Liposome-hCGRP			
	0	12	24	48	0	12	24	48
1	+213	+102	+52	+29	+197	+191	+193	+190
2	+191	+93	+49	+33	+223	+190	+194	+189
3	+187	+114	+79	+22	+186	+187	+185	+181
4	+194	+90	+82	+19	+187	+185	+186	+179
5	+188	+87	+51	+24	+195	+192	+193	+183
mean ±SD	+195 (16.9)	+97 (14.4)	+63 (16.7)	+25 (6.8)	+198 (19.2)	+189 (12.3)	+190 (7.2)	+184 (6.1)

Methods: Experiments were performed on New Zealand white rabbits (weight 2.5-3.5 kg) that were anesthetized with pentobarbital sodium (30mg/kg, i.v.). Rabbits were placed in a headholer, diameters of ocular vessels (conjuctive) were measured by use of a microscope equipped with a TV camera coupled a video monitor. Images were recorded in

computer and vessel diameters were measured later with an image analyzer software. The analytical system of microcirculation was purchased from DAHENG Co. China. 10μl of diluted samples was dropped into the eye of the rabbits and the images of ocular vessels were recorded in a computer. The vessel diameter of the images was analyzed by the microcirculation software.

In H_2O, the vasodilatory activities (% diameter) of free hCGRP and hCGRP reconstituted in liposome membrane of soybean phospholipid were altered from +187±8.9% to +83±12.4% ($p<0.001$, n=5) and from +198±16.4 to +196±14.3% respectively after a storage period of 90 days. In human plasma, their activities decreased from +195±16.49% to +25±6.8% ($p<0.001$, n=5) and from +198±19.2%to +184±6.1% respectively after incubation for 48 hours. These results indicated that the reconstituted hCGRP in the liposome membrane is more stable in comparison with free hCGRP.

Treatment Experiment 1

Role of liposomal hCGRP in treatment of patients with congestive heart failure (CHF)

Patients: The human studies were carried out in sixteen patients admitted for the control of congestive heart failure: seven were male and nine female, with an average age of 66.3 years (range 54 to 75). Six in New York Heart Association (NYHA) phase IV, seven in phase III and three in phase II [Bruce R. A. Mod. Concepts Cardiovasc. Dis. 1956, 25:321-326]. All patients were treated with liposomal hCGRP after stopping treatment with other drug such as digoxin for three days.

Drugs: Liposomal hCGRP, prepared by Example 1, was used for treatment of the patients, containing 20 pg hCGRP / 5ml solution. Drug content is 2000BU/5ml.

Dose administration route:
Mucosal absorption: via oral-nasal mucosal 40-80 BU (1-3 drops) three times per day; via anus2000BU 3 times per day;
Intravenous infusion: 2000-8000BU (2-4 ampoules) of Liposomal hCGRP added to 5% GS or 100-250ml 0.9% NaCl solution. 1 time per day.

Measurement: Before and after taking the drug, breathing rate, vesicular sound, heart rate and rhythm, liver size, swelling index, weight, urinary volume, and cardiac performance by means of ECG, Echocardiography were observed and measured daily.

Result: Liposomal-hCGRP had sustained beneficial effects on patients with CHF. Most patients felt symptomatically better the next morning. There was a dominant cardiac improvement in 9 patients, effective in 6, and only one remained unchanged. No subject complained of side-effect to the drug such as headache, flushing. The drug did not cause hypotension and did not affect the liver or renal function during treatment.

Table 8

Treatment effect of liposomal hCGRP on patients with congestive heart failure						
Patient No.	Pre-drug	OD	Days	Efficiency		
				DE	E	ND
1. CO intoxication	III	i.n	1		II	
2. PCD	IV	i.n	1		III	
3. CO intoxication	III	i.n	1		II	
4. CAD	II	i.n	7			II
5. HCD	II	i.n	7		I	
6. SCD	IV	i.v	7	I		
7. MI	IV	i.v	7		II	
8. MD	III	i.v	7	I		
9. MD	III	i.v	7	I		

Table 8 (continued)

Treatment effect of liposomal hCGRP on patients with congestive heart failure						
Patient No.	Pre-drug	OD	Days	Efficiency		
				DE	E	ND
10. MD	IV	i.v	7		III	
11. MD	III	i.v	7	I		
12. HCD	III	i.v	7	I		
13. HCD	IV	i.v	7	II		
14. HCD	IV	i.v	7		II	
15. HCD	IV	i.v	7	II		
16. HCD	III	i.v	7	I		
* i.n: nasal mucosal administration; iv: intravenous administration; PCD: Pulmonary cardiac disease; CAD: Coronary artery disease; HCD: Hypertensive cardiac disease; MI: Myocardial infarction; MP: Myocardial disease; DE: Dominant efficiency; E: Efficiency; ND: No difference.						

Discussion: Congestive heart failure (CHF) is usually caused by reduced cardiac output as a result of impaired myocardial contractivity, improvement of which is an important object of treatment in patients with CHF. Calcitonin gene related peptide (CGRP) is a neuropeptide with potent vasodilation and positive chronotropic and inotropic action on the heart, indicating that it may be used for CHF treatment. The recent studies have proved that intravenous infusion of CGRP (8.0ng/kg/min) for 8h caused a decrease in right arterial, pulmonary artery, pulmonary artery wedge and systemic arterial pressure.

Cardiac output, stroke volume, and renal blood flow and glomerular filtration increased. Application of liposomal hCGRP in this invention to 16 CHF patients gave beneficial effects, and was characterized by the following:

(1) hCGRP releases gradually from liposomes with long-term effect, have an average effective time of 10 hours, which is 5 fold greater than the results obtained in other investigations;

(2) easy absorption via mucosa, such as oral, nasal and anus administration;

(3) bioavailability of liposomal hCGRP being 10 fold greater than that of hCGRP reported in other investigations.

Treatment Experiment 2

Role of liposomal hCGRP in treatment of patients with essential hypertension

Materials and Method:

Patients: Twenty one patients were hospitalized with essential hypertension of which ten male and eleven female, average age of 62.2 years (rang 45 to 73) and one had aldosteronism. Their hypertension ranged from 3 to 37 years, and their clinical diagnoses based on clinical information was clear. According to WHO/ISH 1993 hypertension diagnosis standard (Beijing Renmin Weigheng Chubanshe 1996, 227-228), patients in phase hypertension were 11, in III hypertension 10.

Drugs and Measurement: Sixteen patients stopped administration of other hypotensive drugs for two weeks, five patients with little hypotension were treated with mepramidil and carvedilol. Liposomal hCGRP, prepared in Example 1, was used for all patients by means of i.v infusion or oral-nasal mucosal administration.

a. Oral-nasal mucosal: 0.05-0.10 ml liposome-hCGRP, containing 0.2-0.4 pg hCGRP, was given three times per day and for five consecutive days.

b. Intravenous infusion: one ampoule of liposome-hCGRP, containing 20 pg hCGRP in 5 ml aqueous solution, was given in 100-500 ml 0.9% NaCl per day and for five preceding days.

c. Measurement: Artery blood pressure (BP) was measured at 15, 30, 60, 120, 180 min after administration on the

first day. In the following days, the BP were recorded 6 times before and after drug administration.

Determination of hypertension-relieving:

According the diagnosis standard of 1979 cardiovascular epidemiology (Henan, Zhen Zhou, China) [J. Chinses Cardiovascular Diseases 1979 7:(2):18], hypotension of liposomal hCGRP was determined.

Dominant efficacy: diastolic pressure decrease > 100 mmHg to normotensive level, or only > 20 mmHg.

Efficacy: diastolic pressure decrease 10 mmHg and to normotensive level, or 10-19 mmHg.

Uneffective: diastolic pressure did not decrease to normotensive level or decrease <10 mmHg.

For patients only with systolic pressure increasing, hypotension of drug was determined according to the above standard, but systolic pressure decreasing should be more than 20 mmHg.

Results: 21 patients were treated with liposomal hCGRP prepared by the present invention, 4 mucosal administration, 4 intravenous infusion, 13 were given combinative administration of i.v. with mucosal, 2 via anius mucosa, 2 via oral mucosa, and rest via nasal mucosa.

Treatment result: Systolic pressure decreased 20-105 mmHg with an average decrease of 17 mmHg ($p<0.001$, $n=21$).

Diastolic pressure was decreased 5-25 mmHg with an average decrease of 17 mmHg ($p<0.001$, $n=21$). Liposomal hCGRP was dominant efficacy for 13 patients, effective for 7 and ineffective for 1. Hypertension-relieving began within 5 minutes after administration, and was maintained approx. 10h.

Secondary effect:

2 patients with chronic nasitis felt a little comfortless by nasal mucosa administration, after i.v. infusion was used, nasal symptom vanished. During treatment with liposomal hCGRP, headache and flushing did not occur, no liver and renal lesions was observed.

Discussion

1. CGRP is endogenous neuropeptide. Liposomal hCGRP in the present invention has avoided rapid degradation of CGRP to achieve long-term effect by gradually releasing hCGRP in vivo, and it is easily absorbed by tissue cells. For treatment of hypertension, it takes effect very quickly, effectively and safely. In 21 patients dominant efficacy achieved 61.9%, total efficiency 95.2%, only one was ineffective, and no remarkable difference was observed for treatment efficiency of hypertension by either i.v. infusion or mucosal absorption of liposomal hCGRP.

2. Some reports indicated dose-dependent effect of CGRP on hypertension in animal with hypotension efficacy increased with increase in dosage. However, in 21 patients with hypertension in the experiment, optimal hypertension-relieving efficacy was observed by 40-80 BU liposomal hCGRP via nasal mucosal absorption, but further increase of the dose did not give a better result, which may be caused by increase of cardiac output induced by hCGRP positive inotropic action on heart.

3. Shekgar et al. [Shekhar YC, et al., Effects of Prolonged Infusion of Human Alpha Calcitonin Gene-Related Peptide on Hemodynamics, Renal Blood Folw and Hormone Levels in Congestive Heart Failure, Am J Cardiol 1991; 67:733.] reported that i.v. infusion of hCGRP (8.0ng/kg/min) for 8h with total dose of 3840ng/kg induced hypotension, systemic artery blood pressure decreased 18% ($p<0.05$) 30 min after drug intake. In this experiment, the hypotensive dose was 8000 BU/day, and hCGRP content was only 780 pg, i.e., 0.8 pg hCGRP per kg body weight, equal to 2.0×10^{-7} fold of the hCGRP dose reported by other investigations.

4. A small secondary effect was noted when 1 patients felt nasal comfortless after nasal administration, possibly related to nasal vasodilation. Thus patients with nasal disease should be treated by other route of administration of liposomal hCGRP.

Claims

1. A pharmaceutical composition of hCGRP, comprising liposomes obtained from natural soybean phospholipid, in which the weight ratio of hCGRP to soybean phospholipid is 1-2 to 100-8000.

2. A pharmaceutical composition of hCGRP as claimed in claim 1, wherein the weight ratio of hCGRP to soybean phospholipid is 1.5-2 to 2500-6000.

3. A pharmaceutical composition of hCGRP as claimed in claim 1 or 2, containing 20-2000pg hCGRP in 5ml of said composition.

4. A method for preparing the pharmaceutical composition of hCGRP as claimed in claim 1, characterized by the following steps:

   (1) adding sterilized and distilled water to purified and dried soybean phospholipid in a weight ratio of lipid to water being greater than 1 to 1000, followed by sonicating to obtain small and single-membrane vesicles of lipid bilayer;

   (2) mixing hCGRP, dissolved in H_2O with the ratio of peptide to H_2O being 1 to 1000-25000, with the above soybean phospholipid in a ratio the peptide to lipid being 1-2 to 100-8000, more particularly 1.5-2 to 2500-6000, sonicating and incubating at 37°C for 30-60 min to obtain a stable composition of liposomal hCGRP.

5. A method as in claim 4, characterized by a weight ratio of hCGRP to soybean phospholipid of 1.5-2 to 2500-6000 in step (2).

6. A method as claimed in claim 4 or 5, characterized by the further step of lyophilizing, and then dissolving the liposomal hCGRP in H_2O to obtain an aqueous solution containing 20-2000pg hCGRP per 5ml solution.

7. A pharmaceutical composition as claimed in any of claims 1 to 3 for use in the treatment of hypertension of humans.

8. A pharmaceutical composition as claimed in any of claims 1 to 3 for use in the treatment of congestive heart failure of humans.

9. A pharmaceutical composition as claimed in claim 7 or 8, wherein it is administered to the patient by intravenous infusion, oral, nasal mucosal spray.

10. A pharmaceutical composition as claimed in any of claims 7 to 9, wherein it is administered to the patient in an amount of 0.1-10 pg hCGRP per kg body weight.

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(54) Liposomal human calcitonin gene-related peptide composition and preparation of the same

(57) The present invention provides a pharmaceutical composition of hCGRP and the preparation of the same. The composition comprises liposomes from natural soybean phospholipid, in which the weight ratio of hCGRP to soybean phospholipid is 1-2 to 100-8000. The half-life of the composition is longer than 72 hours, and the stability of the composition is also elongated. The composition can be administrated by intravenous infusion, oral, nasal mucosal spray in an amount of 0.1-10 pg hCGRP per kg body weigh to treat hypertension and congestive heart failure of a human. The bioavailability of approximately 80%.

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EUROPEAN SEARCH REPORT

Application Number
EP 97 12 0955

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
X	WO 96 03993 A (BULLOCH KAREN ;MCEWEN BRUCE S (US); UNIV ROCKEFELLER (US)) 15 February 1996 * page 6, line 4 - line 6 * * page 10, line 17 - line 18 * * page 12, line 23 - line 25 * * page 17, line 23 - page 18, line 6 *	1-6,10	A61K38/22 A61K9/127
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X,P E	WO 97 06813 A (HISAMITSU PHARMACEUTICAL CO ;YAMAMOTO NAKAYUKI (JP); ITO TERUOMI ()) 27 February 1997 & EP 0 845 265 A (HISAMITSU PHARMACEUTICAL CO ; ASAHI CHEMICAL IND (JP)) 3 June 1998 * abstract; claims 3,9,14,23 *	1-6,10	TECHNICAL FIELDS SEARCHED (Int.Cl.6) A61K C07K
A	EP 0 335 597 A (TOYO JOZO KK ;TOSOH CORP (JP)) 4 October 1989 * page 4, line 6 - line 29 *	4,6	
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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	10 February 1999	Stein, A

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

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ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 97 12 0955

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
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10-02-1999

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82